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Towards Novel 1,2,4,5-Tetrazine Mediated Peptide Macrocyclisations

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Doctor of Philosophy in Chemistry

The University of Edinburgh

2015

Abstract

Interest in linear peptides and peptidomimetics experienced rapid growth within the pharmaceutical industry due to their ability to disrupt protein-protein interactions; and their ability to modulate protein function by allosteric regulation and competitive binding. However, many linear peptides that show therapeutic potential cannot be readily developed into bioavailable pharmaceuticals due to their poor lipophilicity and protease stability; and their unpredictable secondary structures. Macrocyclic peptides show increased lipophilicity and protease stability; and their constrained secondary structures often lead to improved target binding affinity and selectivity. Consequently, there has been a resurgence of interest within the pharmaceutical industry towards peptide based therapeutics and increased research towards novel peptide macrocyclisation strategies.

Progress towards a novel solid-phase peptide macrocyclisation strategy based on the inverse electron demand Diels-Alder reaction of 1,2,4,5-tetrazines is reported in this thesis. The solid-phase oxidation activation peptide macrocyclisation strategy uses the *in situ* oxidation of a dihydro-1,2,4,5-tetrazine to trigger the inverse electron demand Diels-Alder reaction of a 1,2,4,5,-tetrazine and therefore peptide macrocyclisation. Also, the solid-phase oxidation activation peptide macrocyclisation strategy enables selective late stage incorporation of the dihydro-1,2,4,5-tetrazine moiety onto the peptide backbone in the presence of a dienophile. A successful proof of concept *in situ* oxidation and inverse electron demand Diels-Alder reaction of a dihydro-1,2,4,5-tetrazine was achieved. However, the poor stability of a strained dienophile towards an oxidant was also highlighted. To overcome this problem a library of alternative oxidants and a library of unstrained dienophiles were screened to successfully optimise the proof of concept *in situ* oxidation and inverse electron demand Diels-Alder reaction of a dihydro-1,2,4,5-tetrazine. The optimised *in situ* oxidation and inverse electron demand Diels-Alder reaction of a dihydro-1,2,4,5-tetrazine was successfully transferred onto solid-phase using resin-bound dienophiles. However, attempts to synthesise resin-bound dihydro-1,2,4,5-tetrazine derivatised amino acids were unsuccessful. Therefore, an alternative dihydro-1,2,4,5-tetrazine was selected for

future development of the solid-phase oxidation activation peptide macrocyclisation strategy.

Lay Abstract

A linear peptide is a straight molecule which consists of a short chain of amino acids bound together by chemical bonds. Interest in linear peptides experienced rapid growth within the pharmaceutical industry due to their ability to target both non-traditional and traditional pharmaceutical targets. However, many linear peptides which show therapeutic potential do not reach the market due to their inability to cross barriers and their rapid decomposition within the body.

A macrocyclic peptide is a circular peptide that is formed when a chemical bond links two amino acids within a linear peptide together. Macrocyclic peptides can overcome the problems associated with linear peptides whilst showing additional desirable properties for a pharmaceutical molecule. Consequently, there has been a resurgence of interest within the pharmaceutical industry towards peptide based therapeutics and increased research towards novel peptide macrocyclisation strategies. Progress towards a novel peptide macrocyclisation strategy is reported in this thesis.

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Declaration

This thesis has been composed entirely by the author; and the work presented in this thesis is entirely the authors own and has not been submitted for any other degree or professional qualification.

Signed:
Date:

Acknowledgements

Firstly, I would like to thank Professor Mark Bradley for the opportunity to carry out my Doctor of Philosophy (PhD) in his research group; and both Professor Mark Bradley and Dr Lisa Rooney for their guidance and support throughout my PhD.

Secondly, I would like to thank all the members of the Bradley Research Group and the Global Discovery Chemistry Unit, Horsham who were present during my PhD for everything in both the academic and personal environments.

Thirdly, I would like to thank the Engineering and Physical Sciences Research Council and Novartis for my funding; and the staff at The University of Edinburgh and Novartis who made my PhD possible.

Finally, I would to thank my family and friends for their encouragement and support which made it possible for me to reach the end of my PhD.

Abbreviations

°C	degree Celsius
δ	chemical shift
(±)	racemic
$[2M+H]^+$	quasi-molecular cation
$[2M+Na]^+$	quasi-molecular cation
$[M]^{+\cdot}$	molecular radical cation
$[M-H]^-$	quasi-molecular anion
$[M+H]^+$	quasi-molecular cation
$[M+K]^+$	quasi-molecular cation
$[M+Na]^+$	quasi-molecular cation
[Ox]	oxidation
~	approximately
Aaa	generic amino acid residue
Ac	acetyl
All	allyl
aq	aqueous
Ar	generic aromatic group
Arg	arginine residue
Asn	asparagine residue
Asp	aspartic acid residue
$_{ax}$	axial
BCN	bicyclo[6.1.0]non-4-yn-9-ylmethyl
Boc	*tert*-butyloxycarbonyl
bp	boiling point
br	broad
BTI	[bis(trifluoroacetoxy)iodo]benzene
CAN	ammonium cerium(IV) nitrate
CI	chemical ionisation
COD	*cis*,*cis*-1,5-cyclooctadiene
Cy	cyclohexyl

Cys	cysteine residue
d	day or doublet
DBA	*trans*,*trans*-dibenzylideneacetone
DCM	dichloromethane
DCM-d_2	deuterated dichloromethane
dd	doublet of doublets
ddd	doublet of doublets of doublets
Dde	1-(4,4-dimethyl-2,6-dioxocyclohexylidene)ethyl
ddt	doublet of doublets of triplets
DDQ	2,3-dichloro-5,6-dicyano-*p*-benzoquinone
DEAD	diethyl azodicarboxylate
DIC	*N*,*N*`-diisopropylcarbodiimide
DIPEA	*N*,*N*-diisopropylethylamine
DMAP	4-(dimethylamino)pyridine
DMF	*N*,*N*-dimethylformamide
DMSO	dimethyl sulfoxide
DMSO-d_6	deuterated dimethyl sulfoxide
dq	doublet of quartets
$dr_{E:Z}$	diastereomeric ratio
DSC	*N*,*N*`-disuccinimidyl carbonate
durene	1,2,4,5-tetramethylbenzene
E	*entgegen*
EDC	*N*-(3-dimethylaminopropyl)-*N*`-ethylcarbodiimide hydrochloride
EI	electron ionisation
EDG	generic electron donating group
ELSD	evaporative light scattering detector
ESI	electrospray ionisation
Et	ethyl
eq	equatorial
eq	equivalent
EWG	generic electron withdrawing group

FAB	fast atom bombardment
Fmoc	9-fluorenylmethoxycarbonyl
Gln	glutamine residue
Glu	glutamic acid residue
Gly	glycine residue
h	hour
His	histidine residue
HOMO	highest occupied molecular orbital
HPLC	high-performance liquid chromatography
HRMS	high-resolution mass spectroscopy
Hz	Hertz
iedDA	inverse electron demand Diels-Alder
^{i}Pr	isopropyl
IR	infrared
J	nuclear magnetic resonance coupling constant
LDA	lithium diisopropylamide
LG	generic leaving group
LRMS	low-resolution mass spectroscopy
LUMO	lowest unoccupied molecular orbital
Lys	lysine residue
m	multiplet
M	moldm^{-3}
m/z	mass-to-charge ratio
*m*CPBA	3-chloroperbenzoic acid
Me	methyl
Met	methionine residue
min	minute
mL	millilitre
mp	melting point
Ms	methanesulfonyl
n	*primary*
NMR	nuclear magnetic resonance

OSu	*N*-succinimidyl carbonate
oxyma	ethyl (hydroxyimino)cyanoacetate
p	*para*
Pbf	2,2,4,6,7-pentamethyldihydrobenzofuran-5-sulfonyl
PG	generic protecting group
Ph	phenyl
PhD	Doctor of Philosophy
ppm	parts per million
PTAD	4-phenyl-1,2,4-triazoline-3,5-dione
q	quartet
qNMR	quantitative nuclear magnetic resonance
R	generic chemical group
R	*rectus*
R_f	retention factor
Rink amide linker	4-[(2,4-dimethoxyphenyl)(Fmoc-amino)methyl]phenoxyacetic acid
rt	ambient temperature
s	singlet
S	*sinister*
sat	saturated
Ser	serine residue
S_NAr	nucleophilic aromatic substitution
SPPS	solid-phase peptide synthesis
t	triplet
^{t}Bu	*tert*-butyl
TC	thiophene-2-carboxylate
td	triplet of doublets
tert	*tertiary*
Tf	trifluoromethanesulfonate
TFA	trifluoroacetic acid
THF	tetrahydrofuran
Thr	threonine residue

TIS	triisopropylsilane
TLC	thin-layer chromatography
TPAT	trimethylphenylammonium tribromide
t_R	retention time
Trp	tryptophan residue
Trt	triphenylmethyl
tt	triplet of triplets
Tyr	tyrosine residue
Z	*zusammen*

Chapter 1. Introduction

1.1. Peptide based Therapeutics

Interest in linear peptides and peptidomimetics experienced rapid growth within the pharmaceutical industry due to their ability to disrupt protein-protein interactions; and modulate protein function by allosteric regulation and competitive binding.[1,2] Also, linear peptides bridge the gap between small molecule and biologic therapeutics which was a previously unexplored region of chemical space for pharmaceutical molecules. Furthermore, the amino acid building blocks of linear peptides provide chemical diversity and non-toxic metabolites upon proteolysis of a linear peptide. However, many linear peptides that show therapeutic potential cannot be readily developed into bioavailable pharmaceuticals due to their poor lipophilicity and protease stability; and their unpredictable secondary structures. Macrocyclic peptides show increased lipophilicity and protease stability; and their constrained secondary structures often lead to improved target binding affinity and selectivity. Consequently, there has been a resurgence of interest within the pharmaceutical industry towards peptide based therapeutics and increased research towards novel peptide macrocyclisation strategies.

1.2. Synthesis of Macrocyclic Peptides

General strategies for the synthesis of macrocyclic peptides include head-to-side chain, head-to-tail, side chain-to-side chain or side chain-to-tail peptide macrocyclisations (Figure 1).[3] The macrocyclic peptide that is synthesised depends upon the functional groups present on the peptide backbone and the peptide macrocyclisation strategy used for the synthesis of that macrocyclic peptide. Head-to-side chain, head-to-tail, side chain-to-side chain or side chain-to-tail lactamisation; head-to-side chain or side chain-to-side chain lactonisation; and side chain-to-side chain disulfide bridge formation are examples of peptide macrocyclisation strategies which use standard proteinogenic amino acids for the synthesis of macrocyclic peptides.

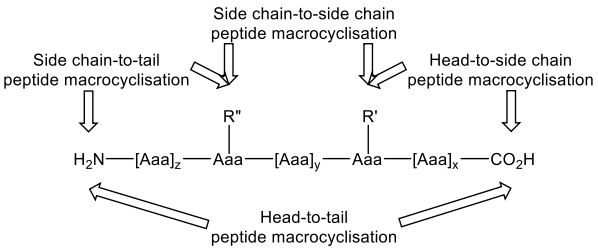
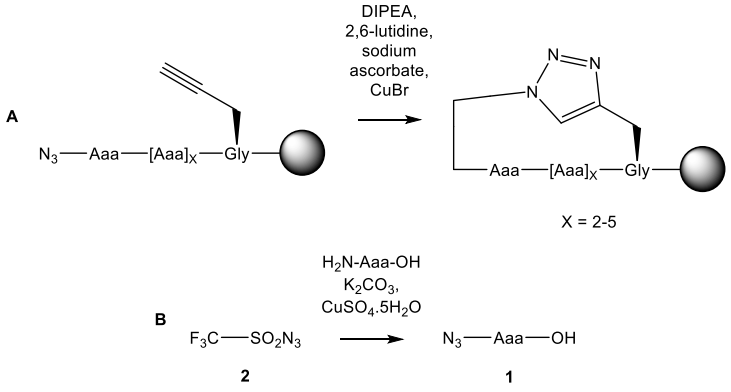


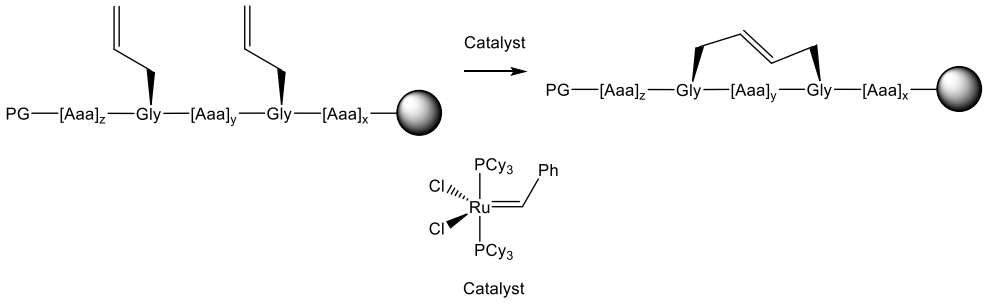
Figure 1. General strategies for the synthesis of macrocyclic peptides.

Side chain-to-tail peptide macrocyclisations of leucine rich linear tetra-, penta-, hexa- and heptapeptides; and ligands of Vascular Endothelial Growth Factor Receptor 1 have been achieved using the solid-phase copper(I) catalysed 1,3-dipolar cycloaddition of an alkyne with an azide peptide macrocyclisation strategy (Scheme 1A).[4-7] However, depending on the linear peptides length and sequence competing intermolecular reactions can give rise to side products during peptide macrocyclisations using the solid-phase copper(I) catalysed 1,3-dipolar cycloaddition of an alkyne with an azide peptide macrocyclisation strategy.[8] The solid-phase copper(I) catalysed 1,3-dipolar cycloaddition of an alkyne with an azide peptide macrocyclisation strategy uses N_3-Aaa-OH **1** and commercially available *L*-*C*-propargylglycine to enable peptide macrocyclisation. The established synthesis of N_3-Aaa-OH **1** is the copper(I) catalysed diazo transfer of trifluoromethanesulfonate azide **2** with H_2N-Aaa-OH in the presence of potassium carbonate (Scheme 1B).[9,10]



Scheme 1A. Solid-phase copper(I) catalysed 1,3-dipolar cycloaddition of an alkyne with an azide peptide macrocyclisation strategy.[6,7] **Scheme 1B.** Synthesis of N_3-Aaa-OH **1**.[10]

Side chain-to-side chain peptide macrocyclisations have been achieved using the solid-phase ruthenium(IV) catalysed ring closing alkene metathesis peptide macrocyclisation strategy (Scheme 2).[11-13] However, incomplete peptide macrocyclisation can be observed during solid-phase ruthenium(IV) catalysed ring closing alkene metathesis peptide macrocyclisation. The solid-phase ruthenium(IV) catalysed ring closing alkene metathesis peptide macrocyclisation strategy uses commercially available *L*-allylglycine to enable peptide macrocyclisation.



Scheme 2. Solid-phase ruthenium(IV) catalysed ring closing alkene metathesis peptide macrocyclisation strategy.[13]

Many other examples of peptide macrocyclisation strategies which use non-proteinogenic amino acids have been reported within the literature.[3] However, each peptide macrocyclisation strategy reported within the literature has associated limitations. Therefore, further peptide macrocyclisation strategies which are complimentary to the existing peptide macrocyclisation strategies are required. A peptide macrocyclisation strategy based on the inverse electron demand Diels-Alder (iedDA) reaction of 1,2,4,5-tetrazines has not been reported within the literature.[14,15]

1.3. 1,2,4,5-Tetrazines

1,2,4,5-Tetrazines are six-membered azaaromatic compounds which contain two sp^2 hybridised carbon atoms and four sp^2 hybridised nitrogen atoms and can be either symmetrically or unsymmetrically (di)substituted (Figure 2). A diverse range of symmetrically and unsymmetrically (di)substituted 1,2,4,5-tetrazines have been reported within the literature with acyl, alkenyl, alkyl, alkynyl, amide, aryl, ester,

halogen, heteroaryl and heteroatom substituents.[16-25] The first reported synthesis of a disubstituted 1,2,4,5-tetrazine was in 1893 whilst the first reported synthesis of 1,2,4,5-tetrazine was in 1900.[21,26]

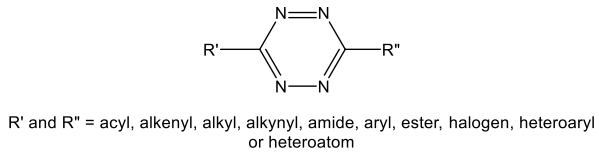


Figure 2. 1,2,4,5-Tetrazines.

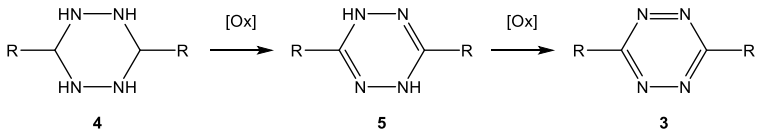
Due to the presence of four electronegative nitrogen atoms 1,2,4,5-tetrazines can be considered as the most electron deficient parent azaaromatic compound as both pentazine and hexazine are unstable at ambient temperature (rt).[27] The electron deficient nature of 1,2,4,5-tetrazines enables them to be reduced at high potentials and leads to a low-lying π* molecular orbital.[28] The low-lying π* molecular orbital gives rise to their characteristic purple or red colours due to a weak n-π* transition in the visible region of the electromagnetic spectrum.[29] The n-π* transition of 1,2,4,5-tetrazines is weakly dependent upon the nature of the substituents.[30] Some 1,2,4,5-tetrazines are known to be fluorescent although initially high quantum yields of decomposition were observed upon irradiation.[31,32] The fluorescent properties of 1,2,4,5-tetrazines are dependent upon the size and nature of the substituents.[33,34]

Since the first reported syntheses of 1,2,4,5-tetrazines they have been investigated in numerous fields of research. The coordination chemistry of 1,2,4,5-tetrazines has been researched due to their metal-to-metal bridging capacity which can be exploited in functional supramolecular structures whilst the coordination chemistry; and electrochemistry and photochemistry of 1,2,4,5-tetrazines has been exploited in catalysis.[35-37] The electrochemistry and photochemistry of 1,2,4,5-tetrazines has been researched to enable the development of novel functional materials and molecules with potential applications in electronics, energy storage, nonlinear optics and sensors.[38]Also, 1,2,4,5-tetrazines have been researched as high-nitrogen energetic materials and have been used in explosives, fire retardants, propellants and pyrotechnics due to their high nitrogen content, heats of formation and thermal

stability.[39-42] Furthermore, the biological activity of 1,2,4,5-tetrazines has been researched and they have been used in crop protection and show potential as pharmaceutical molecules.[43,44]

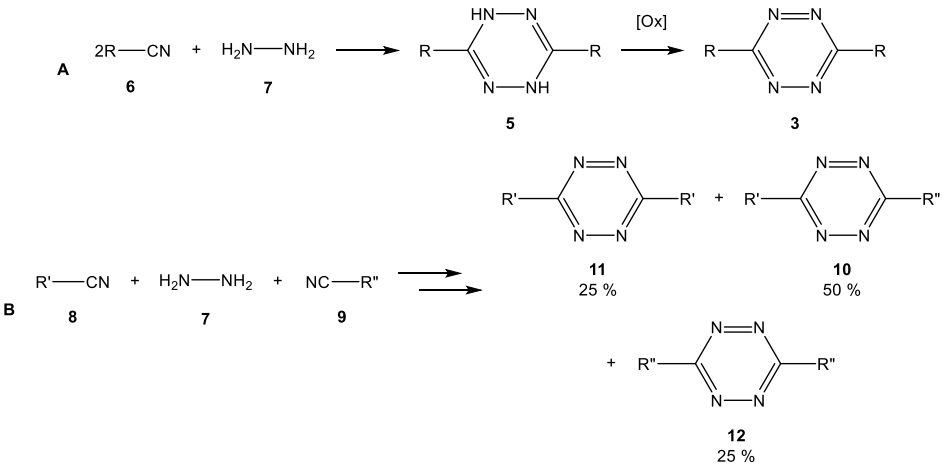
1.3.1. Synthesis of 1,2,4,5-Tetrazines

There are no synthetic routes which enable the direct synthesis of the 1,2,4,5-tetrazine core. Therefore, all synthetic routes towards symmetrically disubstituted tetrazine **3** must synthesise and subsequently oxidise symmetrically disubstituted tetrahydrotetrazine **4** or symmetrically disubstituted dihydrotetrazine **5** (Scheme 3). Additional 1,2,4,5-tetrazines can be synthesised by the modification or substitution of existing substituents on the 1,2,4,5-tetrazine core.



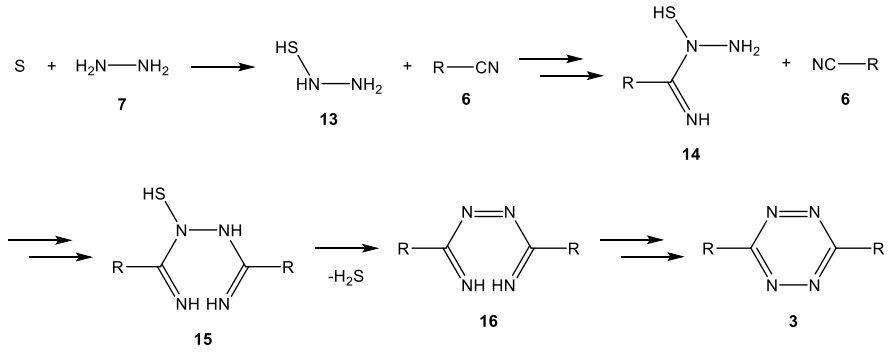
Scheme 3. Synthesis of symmetrically disubstituted tetrazine **3**.

The established synthesis of diaryl 1,2,4,5-tetrazines is the Pinner synthesis of disubstituted 1,2,4,5-tetrazines (Scheme 4A).[21] The reaction of nitrile **6** with hydrazine **7** and subsequent oxidation of symmetrically disubstituted dihydrotetrazine **5** gives symmetrically disubstituted tetrazine **3**. The reaction of nitriles **8** and **9** with hydrazine **7** and subsequent oxidation of the resulting unsymmetrically disubstituted dihydro-1,2,4,5-tetrazine gives unsymmetrically disubstituted tetrazine **10** (Scheme 4B). However, the Pinner synthesis of unsymmetrically disubstituted 1,2,4,5-tetrazines only gives unsymmetrically disubstituted tetrazine **10** at 50 % theoretical yield due to side reactions giving symmetrically disubstituted tetrazines **11** and **12** at 25 % theoretical yield respectively. An additional limitation of the Pinner synthesis of disubstituted 1,2,4,5-tetrazines is the poor tolerance to alkyl nitriles when attempting to synthesise disubstituted 1,2,4,5-tetrazines with alkyl substituents.[45] Also, the formation of disubstituted 1,2,4-triazole-4-amine side products has been reported within the literature during the Pinner synthesis of disubstituted 1,2,4,5-tetrazines.[46,47]



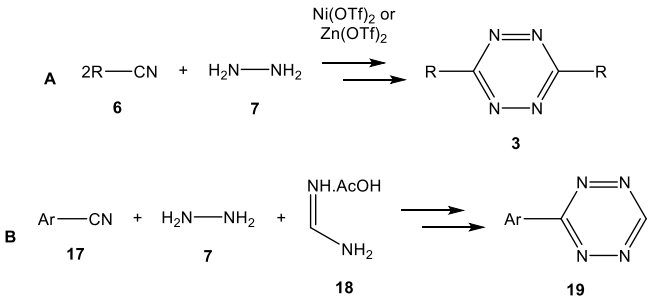
Scheme 4A. Pinner synthesis of symmetrically disubstituted tetrazine **3**.[21] **Scheme 4B.** Pinner synthesis of unsymmetrically disubstituted tetrazine **10**.

No mechanism has been proposed within the literature for the Pinner synthesis of disubstituted 1,2,4,5-tetrazines. However, a mechanism has been proposed within the literature but not corroborated by any mechanistic studies for the sulfur-promoted Pinner synthesis of disubstituted 1,2,4,5-tetrazines (Scheme 5).[48,49] The addition of sulfur to hydrazine **7** and subsequent nucleophilic addition of nitrile **6** to thiol hydrazine **13** was proposed to give thiol amidrazone **14**. Successive nucleophilic addition of nitrile **6** to thiol amidrazone **14** and subsequent elimination of hydrogen sulfide from thiol dicarboximidamide **15** was proposed to give diimine azo **16**. The ring closure of diimine azo **16** and subsequent oxidation of the resulting symmetrically disubstituted dihydro-1,2,4,5-tetrazine was proposed to give symmetrically disubstituted tetrazine **3**. Evidence for the proposed mechanism was given by a colour change upon the addition of sulfur to hydrazine **7** and the elimination of hydrogen sulfide during the sulfur-promoted Pinner synthesis of disubstituted 1,2,4,5-tetrazines.



Scheme 5. Proposed mechanism for the sulfur-promoted Pinner synthesis of symmetrically disubstituted tetrazine **3**.[49]

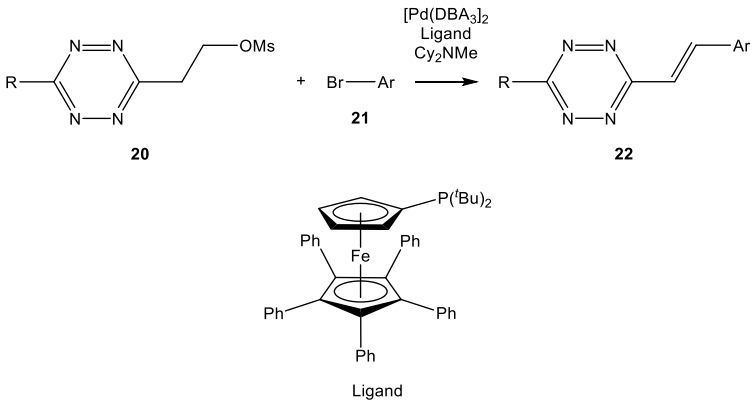
The established synthesis of disubstituted 1,2,4,5-tetrazines with alkyl substituents is the Lewis acid catalysed Pinner synthesis of disubstituted 1,2,4,5-tetrazines (Scheme 6A).[18] A limitation of the Lewis acid catalysed Pinner synthesis of disubstituted 1,2,4,5-tetrazines is the requirement to use anhydrous hydrazine which has limited commercial availability due to its use as rocket fuel. No mechanism has been proposed within the literature for the Lewis acid catalysed Pinner synthesis of disubstituted 1,2,4,5-tetrazines. However, it was proposed in the literature that the Lewis acid might promote the nucleophilic addition of nitrile **6** to hydrazine **7**. It was also reported in the literature that Lewis acids significantly improve the isolated yields of the modified Pinner synthesis of unsymmetrical aryl 1,2,4,5-tetrazines (Scheme 6B).[50] The reaction of aryl nitrile **17** with hydrazine **7** and carboximidamide salt **18** and subsequent oxidation of the resulting unsymmetrical aryl dihydro-1,2,4,5-tetrazine gives unsymmetrical aryl tetrazine **19**.



Scheme 6A. Lewis acid catalysed Pinner synthesis of symmetrically disubstituted tetrazine **3**.[18]

Scheme 6B. Modified Pinner synthesis of unsymmetrical aryl tetrazine **19**.[50]

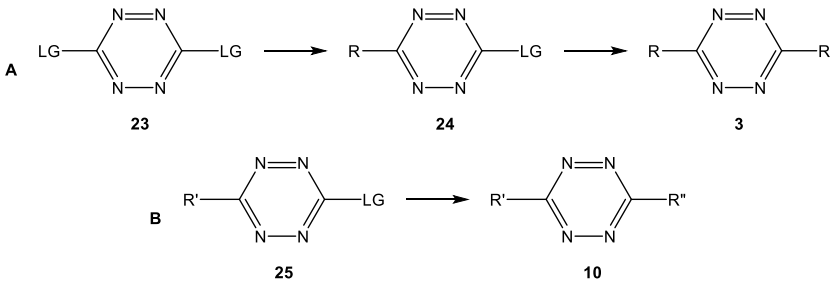
The first synthetic route for the synthesis of a disubstituted 1,2,4,5-tetrazine with alkenyl substituents was developed to enable the synthesis of diethenyl-1,2,4,5-tetrazine.[17] However, the *in situ* elimination and Heck cross-coupling reaction of unsymmetrical methanesulfonyl protected tetrazine **20** with bromoaryl **21** to give unsymmetrical alkenyl tetrazine **22** enables the synthesis of disubstituted 1,2,4,5-tetrazines with alkenyl substituents (Scheme 7).[51]



Scheme 7. Synthesis of unsymmetrical alkenyl tetrazine **22**.[51]

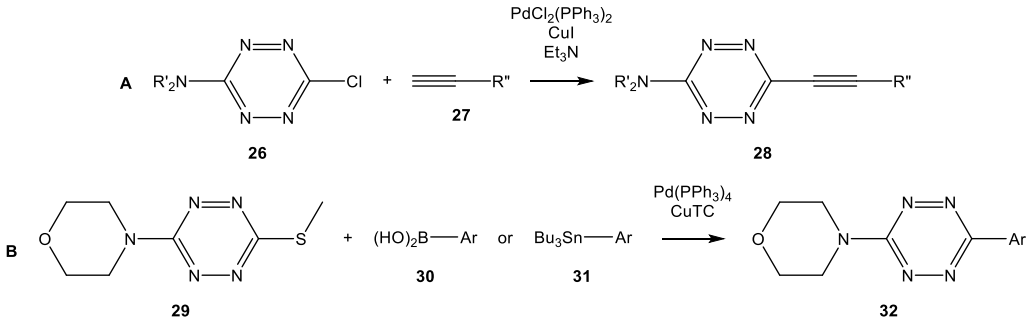
The electron deficient nature of 1,2,4,5-tetrazines enables them to undergo nucleophilic aromatic substitution (S_NAr) when suitable leaving groups are present on the 1,2,4,5-tetrazine core (Scheme 8A). The S_NAr of symmetrically disubstituted tetrazine **23** with an appropriate nucleophile gives unsymmetrically disubstituted tetrazine **24** or symmetrically disubstituted tetrazine **3**. Further S_NAr of unsymmetrically disubstituted tetrazine **25** with an appropriate nucleophile gives unsymmetrically substituted tetrazine **10** (Scheme 8B). The outcome of the S_NAr of 1,2,4,5-terazines is dependent upon the nature of the nucleophile and the substituents on the 1,2,4,5-tetrazine core.[52] The most commonly used 1,2,4,5-tetrazines for S_NAr chemistry are dichloro-1,2,4,5-tetrazine, bis(3,5-dimethyl-1*H*-pyrazol-1-yl)-1,2,4,5-tetrazine and bis(methylsulfanyl)-1,2,4,5-tetrazine.[23-25] Heteroatom-based nucleophiles are the most commonly used nucleophiles for the S_NAr of 1,2,4,5-tetrazines whilst carbon-based nucleophiles give azaphilic addition, metal complexation or 1,2,4,5-tetrazine reduction.[53,54] However, the S_NAr of 1,2,4,5-tetrazines with a limited number of carbon-based nucleophiles has been reported

within the literature.[38,55,56] Also, the S_NAr of bis(3,5-dimethyl-1*H*-pyrazol-1-yl)-1,2,4,5-tetrazine with resin-bound cysteine residues has been reported within the literature to give macrocyclic peptides which enabled investigations into the early conformation dynamics of peptides and proteins.[57]



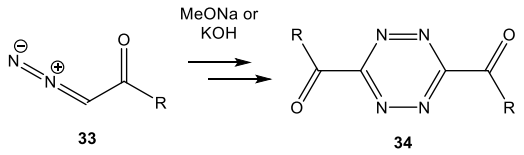
Scheme 8A. Synthesis of unsymmetrically disubstituted tetrazine **24** or symmetrically disubstituted tetrazine **3**. **Scheme 8B.** Synthesis of unsymmetrically disubstituted tetrazine **10**.

The established synthesis of unsymmetrically disubstituted 1,2,4,5-tetrazines with alkynyl substituents is the Sonogashira cross-coupling reaction of amino chloro tetrazine **26** with terminal alkyne **27** to give amino alkynyl tetrazine **28** (Scheme 9A).[19] The palladium(II) catalysed cross-coupling reactions of amino thioester tetrazine **29** with boronic acid **30** or organotin derivative **31** to give amino aryl tetrazine **32** have also been demonstrated within the literature (Scheme 9B).[58] The major limitation of the cross-coupling reactions used for the synthesis of unsymmetrically disubstituted 1,2,4,5-tetrazines with alkynyl substituents is the requirement to use unsymmetrically disubstituted 1,2,4,5-tetrazines with tertiary amine substituents. Unsymmetrically disubstituted 1,2,4,5-tetrazines with primary or secondary amine substituents did not undergo Sonogashira cross-coupling whilst more electron deficient unsymmetrically disubstituted 1,2,4,5-tetrazines decomposed under the reaction conditions.



Scheme 9A. Synthesis of amino alkynyl tetrazine **28**.[19] **Scheme 9B.** Synthesis of amino aryl tetrazine **32**.[58]

The established synthesis of symmetrical diacyl 1,2,4,5-tetrazines is the base promoted dimerisation of diazo ketone **33** and subsequent oxidation of the resulting symmetrical diacyl dihydro-1,2,4,5-tetrazine to give symmetrical diacyl tetrazine **34** (Scheme 10).[16]



Scheme 10. Synthesis of symmetrical diacyl tetrazine **34**.[16]

1.3.2. Inverse Electron Demand Diels-Alder Reaction of 1,2,4,5-Tetrazines

The Diels-Alder reaction is a [4+2] cycloaddition reaction between a diene and a dienophile which is dictated by the highest occupied molecular orbital (HOMO) of the diene and the lowest unoccupied molecular orbital (LUMO) of the dienophile (Figure 3A). Dienes with electron donating groups and dienophiles with electron withdrawing groups increase the rate of reaction of the Diels-Alder reaction by decreasing the $HOMO_{diene}$-$LUMO_{dienophile}$ energy gap. The electron deficient nature of 1,2,4,5-tetrazines enables them to act as azadienes in iedDA reactions.[14,15] The iedDA reaction is dictated by the $HOMO_{dienophile}$ and the $LUMO_{diene}$ (Figure 3B). Dienes with electron withdrawing groups and dienophiles with electron donating groups increase the rate of reaction of the iedDA reaction by decreasing the $HOMO_{dienophile}$-$LUMO_{diene}$ energy gap.

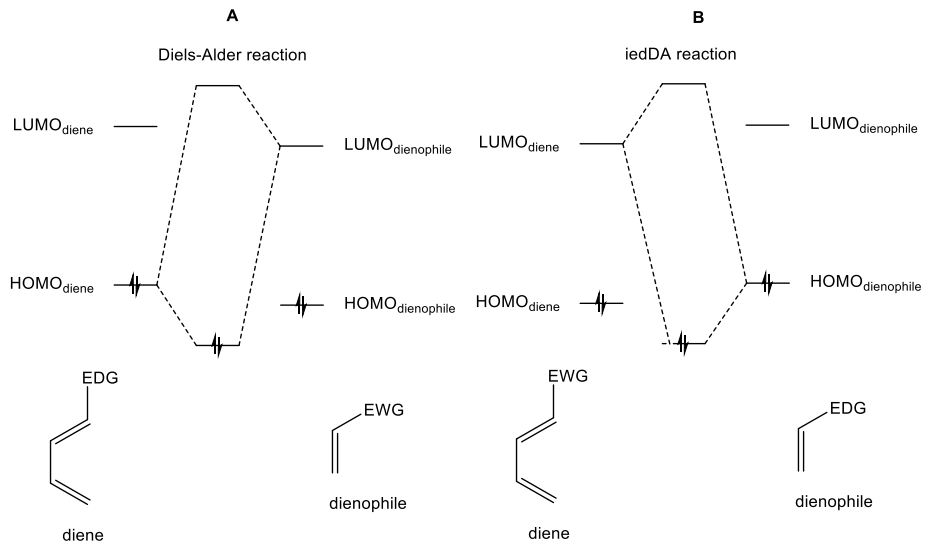
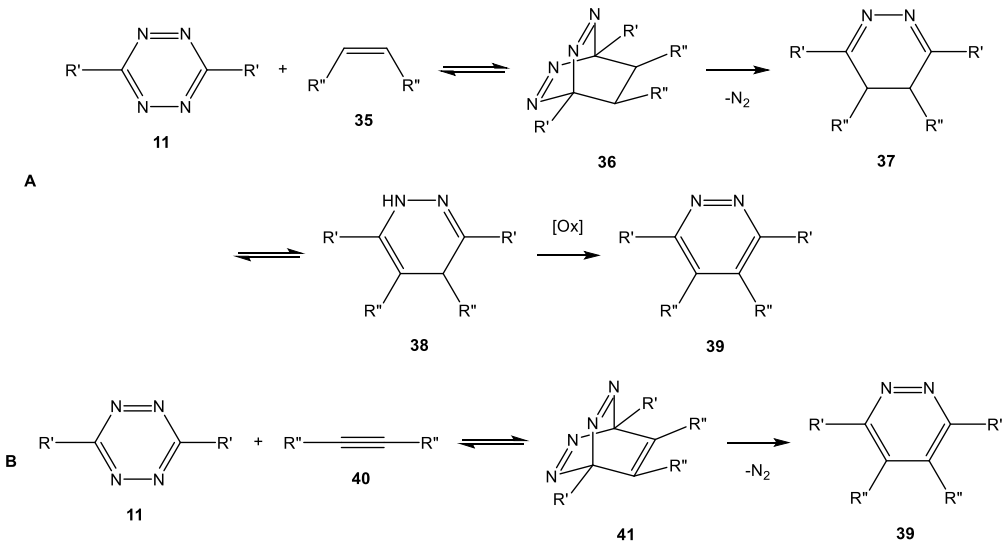


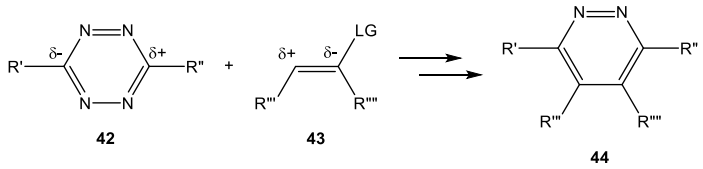
Figure 3A. Diels-Alder reaction molecular orbital diagram. **Figure 3B.** iedDA reaction molecular orbital diagram.

A mechanism was proposed within the literature for the iedDA reaction of 1,2,4,5-tetrazines (Scheme 11A).[14,59] The [4+2] cycloaddition of symmetrically disubstituted tetrazine **11** with symmetrically disubstituted alkene **35** gives bicyclic adduct **36**. Successive *retro*-[4+2] cycloaddition of bicyclic adduct **36** and subsequent tautomerisation of symmetrically tetrasubstituted dihydropyridazine **37** gives symmetrically tetrasubstituted dihydropyridazine **38**.[60] The oxidation of symmetrically tetrasubstituted dihydropyridazine **38** gives symmetrically tetrasubstituted pyridazine **39**. The [4+2] cycloaddition of symmetrically disubstituted tetrazine **11** with symmetrically disubstituted alkyne **40** gives symmetrically tetrasubstituted pyridazine **39** after successive *retro*-[4+2] cycloaddition of bicyclic adduct **41** (Scheme 11B). The rate limiting step of the iedDA reaction of 1,2,4,5-tetrazines is the initial [4+2] cycloaddition with the dienophile.[61] Therefore, iedDA reactions of 1,2,4,5-tetrazines are characterised by second order reaction kinetics. Evidence for a concerted [4+2] cycloaddition is given by large negative entropies of activation and minimal solvent effects on the iedDA reaction of 1,2,4,5-tetrazines.[62] Further evidence for a concerted [4+2] cycloaddition was given by an *ab initio* quantum mechanical investigation into the iedDA reactions of 1,2,4,5-tetrazines.[63] *Ab initio* quantum mechanical calculations on the iedDA reactions of 1,2,4,5-tetrazines suggest that the *retro*-[4+2] cycloaddition of the bicyclic adduct is stepwise.[64]



Scheme 11A. Mechanism of the iedDA reaction of symmetrically disubstituted tetrazine **11** with symmetrically disubstituted alkene **35**. **Scheme 11B.** Mechanism of the iedDA reaction of symmetrically disubstituted tetrazine **11** with symmetrically disubstituted alkyne **40**.

Regioselective iedDA reactions of electronically biased 1,2,4,5-tetrazines with electronically biased dienophiles in solution- and on solid-phase have been reported within the literature (Scheme 12).[65-68] The iedDA reaction of electronically biased tetrazine **42** with electronically biased alkene **43** and subsequent elimination of the leaving group from the resulting unsymmetrically tetrasubstituted dihydropyridazine gives unsymmetrically tetrasubstituted pyridazine **44**. However, iedDA reactions of electronically biased 1,2,4,5-tetrazines with electronically unbiased alkenes have been reported to give a mixture of diastereomeric and regioisomeric dihydropyridazines (Appendix 1).[69] The oxidation of diasteromeric dihydropyridazines gives diastereomerically pure pyridazines due to the aromatic and therefore planar pyridazine core. However, the oxidation of regioisomeric dihydropyridazines gives regioisomeric pyridazines.



Scheme 12. iedDA reaction of electronically biased tetrazine **42** with electronically biased alkene **43**.

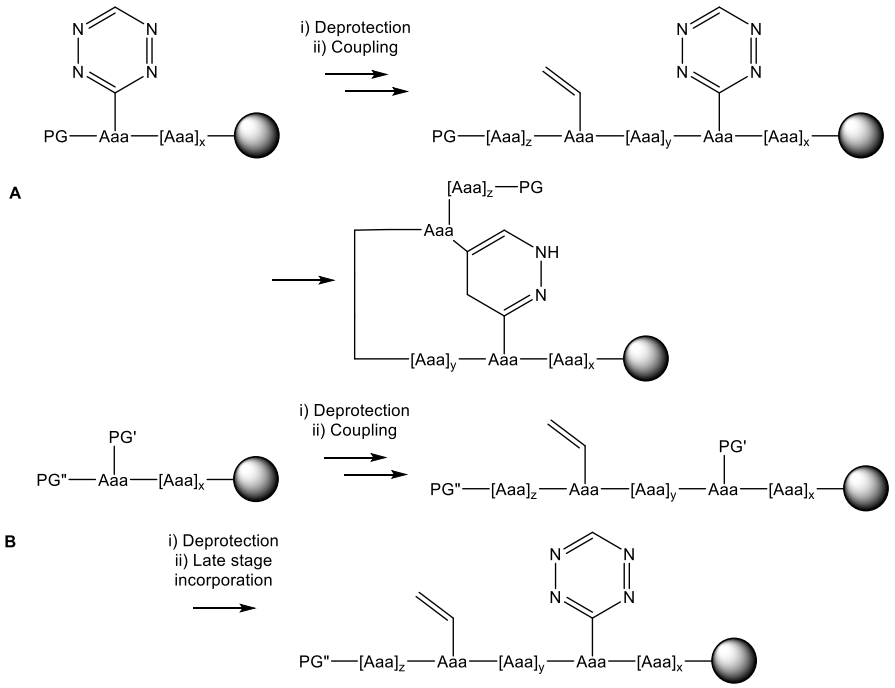
The iedDA reaction of 1,2,4,5-tetrazines undergoes a dramatic increase in the rate of reaction when conducted in hydrous solvents due to hydrogen bonding and hydrophobic effects which stabilise the initial [4+2] cycloaddition transition state.[70-74] The increased rate of reaction observed in hydrous solvents and the bioorthogonality of the iedDA reaction of 1,2,4,5-tetrazines enables it to be used in bioconjugation strategies; and in *in vitro* and *in vivo* imaging.[69,75,76] The iedDA reaction of 1,2,4,5-tetrazines has been considered to fulfil the criteria of a click reaction and therefore has found applications in material, polymer and supramolecular science.[77-83] Further applications of the iedDA reaction of 1,2,4,5-tetrazines include targeted drug delivery, biomolecule immobilisation and natural product synthesis.[84-86]

Chapter 2. Aims

2.1. Aims

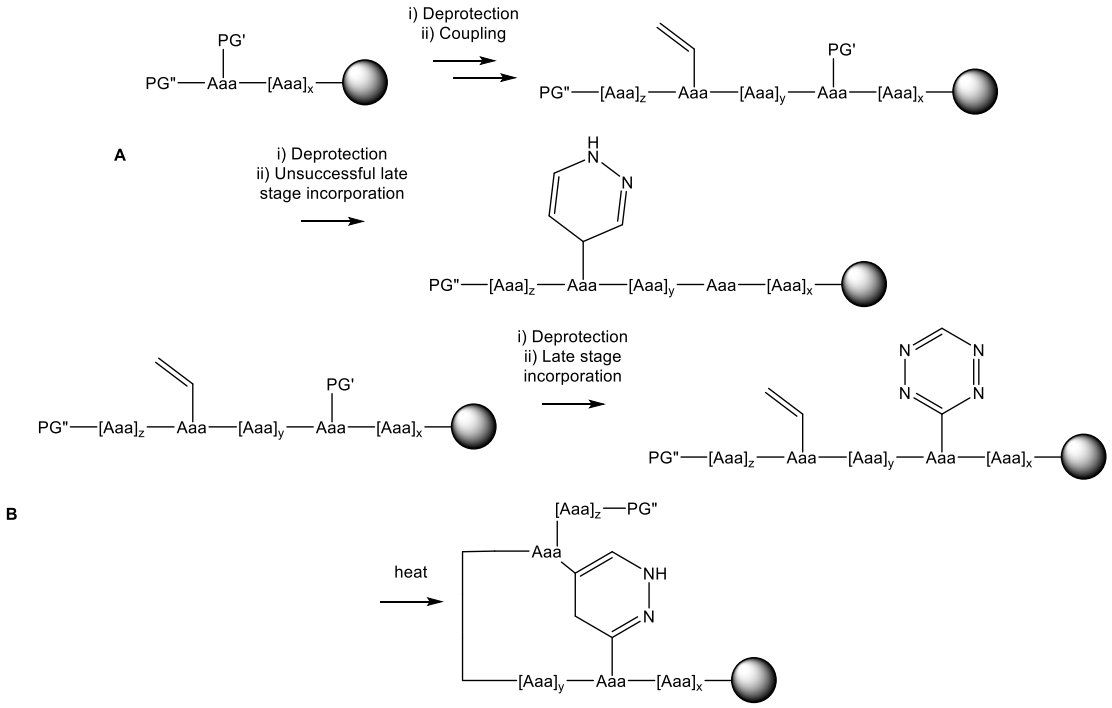
The aim of this thesis was to develop a novel solid-phase peptide macrocyclisation strategy based on the iedDA reaction of 1,2,4,5-tetrazines. A solid-phase peptide macrocyclisation strategy was selected due to their amenability to automated and combinatorial synthesis; and efficiency and simplicity over solution-phase strategies.[87-89] Also, the pseudodilution effect can be exploited to favour intramolecular over intermolecular reactions in a solid-phase peptide macrocyclisation strategy.[90-92] The iedDA reaction of 1,2,4,5-tetrazines was selected due to its efficiency, orthogonality, tunability and versatility as shown by its successful application in numerous fields of research.[69,75,76,79-86]

It was initially proposed that 1,2,4,5-tetrazine and dienophile derivatised amino acids would be incorporated into propagating peptide chains to enable side chain-to-side chain and side chain-to-tail peptide macrocyclisations (Scheme 13A). However, due to the then unknown stability of 1,2,4,5-tetrazines towards the deprotection, coupling and cleavage cycles of solid-phase peptide synthesis (SPPS) it was decided to use an orthogonal deprotection strategy to enable late stage incorporation of the 1,2,4,5-tetrazine moiety onto the peptide backbone (Scheme 13B).[93]



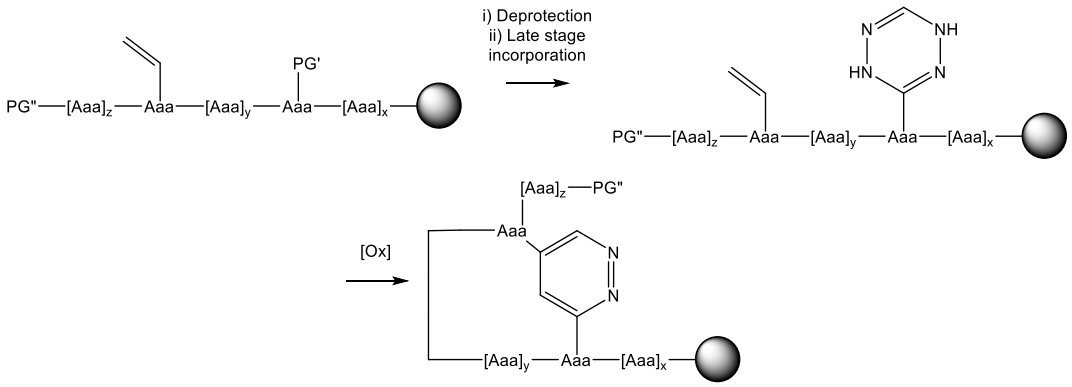
Scheme 13A. 1,2,4,5-Tetrazine and dienophile derivatised amino acids incorporated into a propagating peptide chain to enable a side chain-to-side chain peptide macrocyclisation. **Scheme 13B.** Orthogonal deprotection strategy to enable late stage incorporation of the 1,2,4,5-tetrazine moiety onto the peptide backbone.

A strategy was required to prevent any undesired iedDA reaction during the late stage incorporation of the 1,2,4,5-tetrazine moiety onto the peptide backbone in the presence of a dienophile (Scheme 14A). The solid-phase thermal activation peptide macrocyclisation strategy would use unreactive 1,2,4,5-tetrazine-dienophile pairs to enable late stage incorporation of the 1,2,4,5-tetrazine moiety onto the peptide backbone in the presence of a dienophile at rt (Scheme 14B). Conventional or microwave heating would then increase the rate of reaction to promote the iedDA reaction of the 1,2,4,5-tetrazine and therefore peptide macrocyclisation. However, the fine balance of reaction rates required for selective late stage incorporation of the 1,2,4,5-tetrazine moiety onto the peptide backbone in the presence of a dienophile and complete control over peptide macrocyclisation would be difficult to realise.



Scheme 14A. Undesired iedDA reaction during the late stage incorporation of the 1,2,4,5-tetrazine moiety onto the peptide backbone in the presence of a dienophile. **Scheme 14B.** Solid-phase thermal activation peptide macrocyclisation strategy.

An alternative strategy which would eliminate any competing iedDA reaction during the late stage incorporation of the 1,2,4,5-tetrazine moiety onto the peptide backbone in the presence of a dienophile was proposed. The solid-phase oxidation activation peptide macrocyclisation strategy would use the *in situ* oxidation of an unreactive dihydro-1,2,4,5-tetrazine to trigger the iedDA reaction and therefore peptide macrocyclisation (Scheme 15). The solid-phase oxidation activation peptide macrocyclisation strategy enables selective late stage incorporation of the dihydro-1,2,4,5-tetrazine moiety onto the peptide backbone in the presence of a dienophile and complete control over peptide macrocyclisation.



Scheme 15. Solid-phase oxidation activation peptide macrocyclisation strategy.

The solid-phase oxidation activation peptide macrocyclisation strategy was selected due to its advantages over the solid-phase thermal activation peptide macrocyclisation strategy. An additional advantage of the solid-phase oxidation activation peptide macrocyclisation strategy is that reactive 1,2,4,5-tetrazine-dienophile pairs can be used to enable fast rates of reaction. Also, oxidation of dihydropyridazine diastereomers with excess oxidant will give diastereomerically pure pyridazine and therefore diastereomerically pure macrocyclic peptides. However, to successfully develop a novel solid-phase peptide macrocyclisation strategy based on the *in situ* oxidation and iedDA reaction of a dihydro-1,2,4,5-tetrazine several prerequisites needed to be achieved.

Firstly, a proof of concept *in situ* oxidation and iedDA reaction of a dihydro-1,2,4,5-tetrazine needed to be conducted to determine the viability of the solid-phase oxidation activation peptide macrocyclisation strategy.

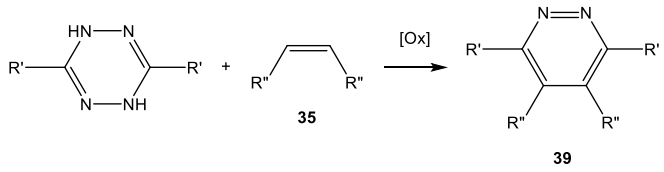
Secondly, optimisation of the proof of concept *in situ* oxidation and iedDA reaction of a dihydro-1,2,4,5-tetrazine would be required before transferring the optimised *in situ* oxidation and iedDA reaction of a dihydro-1,2,4,5-tetrazine to solid-phase and verifying the *in situ* oxidation and iedDA reactions of resin-bound dihydro-1,2,4,5-tetrazine derivatised amino acids.

Finally, peptide macrocyclisations needed to be conducted to determine the scope and limitations of the solid-phase oxidation activation peptide macrocyclisation strategy.

Chapter 3. Proof of Concept

3.1. Aims

The aim of this chapter was to determine the viability of the solid-phase oxidation activation peptide macrocyclisation strategy by conducting a proof of concept *in situ* oxidation and iedDA reaction of a dihydro-1,2,4,5-tetrazine (Scheme 16). However, to achieve a successful proof of concept *in situ* oxidation and iedDA reaction of a dihydro-1,2,4,5-tetrazine several prerequisites needed to be fulfilled.



Scheme 16. *In situ* oxidation and iedDA reaction of a dihydro-1,2,4,5-tetrazine.

Firstly, a dihydro-1,2,4,5-tetrazine which can be readily oxidised to a reactive 1,2,4,5-tetrazine with an oxidant which is amenable to SPPS needed to be identified and synthesised. Also, the dihydro-1,2,4,5-tetrazine required a functional handle to enable late stage incorporation of the dihydro-1,2,4,5-tetrazine moiety onto the peptide backbone.

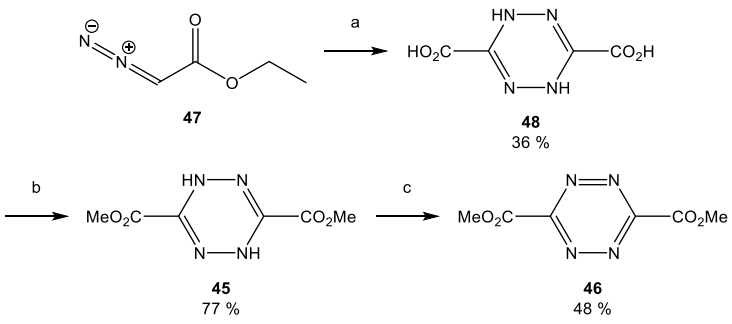
Secondly, a library of reactive dienophiles needed to be identified and synthesised to enable multiple 1,2,4,5-tetrazine-dienophile pairs to be screened. These dienophiles required a functional handle to enable amino acid derivatisation; and the resulting amino acids needed to be stable to the deprotection, coupling and cleavage cycles of SPPS.

Finally, the iedDA reactions of the 1,2,4,5-tetrazine-dienophile pairs needed to be screened before conducting a proof of concept *in situ* oxidation and iedDA reaction of a dihydro-1,2,4,5-tetrazine.

3.2. Selection and Synthesis of Dihydro-1,2,4,5-tetrazine

Diester dihydrotetrazine **45** was selected for the proof of concept *in situ* oxidation and iedDA reaction of a dihydro-1,2,4,5-tetrazine as its synthesis had been previously reported within the literature;[22] it can be readily oxidised to the reactive diester tetrazine **46** with an oxidant which is amenable to SPPS;[80] and it contains two functional handles for the late stage incorporation of diester dihydrotetrazine **45** onto the peptide backbone and further derivatisation.[20]

The established synthesis of diester dihydrotetrazine **45** was developed to enable a large-scale and reproducible synthesis of diester tetrazine **46**.[22] The dimerisation of diazo ester **47** in the presence of sodium hydroxide and subsequent protonation of the resulting sodium salt to give dicarboxylic acid dihydrotetrazine **48** was reported at 69-76 % yield. Successive esterification of dicarboxylic acid dihydrotetrazine **48** with methanol in the presence of thionyl chloride to give diester dihydrotetrazine **45** was reported at 56-61 % yield. However, upon following the literature procedure diester dihydrotetrazine **45** was isolated in 15 % overall yield (Scheme 17). No optimisation was conducted to the literature procedure as sufficient material was obtained for the synthesis of diester tetrazine **46** and the proof of concept *in situ* oxidation and iedDA reaction of a dihydro-1,2,4,5-tetrazine.



Scheme 17. Synthesis of diester tetrazine **46**. Reagents and reaction conditions a) i) NaOH (4.6 eq), H_2O, 70 °C, 1.5 h; ii) HCl, H_2O, -10 °C;[22] b) $SOCl_2$ (2.6 eq), MeOH, 40 °C, 2 h, N_2;[22] c) isopentyl nitrite (3 eq), DCM, rt, 2 h.[80]

The oxidation of diester dihydrotetrazine **45** to give diester tetrazine **46** was reported at quantitative yield.[22] However, the oxidation of diester dihydrotetrazine **45** was achieved with highly toxic nitrous gasses, generated in a separate reaction vessel by the disproportionation of nitrous acid, which are not amenable to SPPS. An alternative oxidation procedure which is amenable to SPPS used isopentyl nitrite to oxidise diester dihydrotetrazine **45** but was reported in the literature without a yield.[80] Upon following the literature procedure diester tetrazine **46** was isolated in 48 % yield (Scheme 17). However, evidence of quantitative oxidation of diester dihydrotetrazine **45** with isopentyl nitrite was obtained by proton nuclear magnetic resonance (NMR) as no starting material, decomposition or side products were observed in the crude material. Therefore, it was concluded that isopentyl nitrite was a suitable oxidant for the proof of concept *in situ* oxidation and iedDA reaction a dihydro-1,2,4,5-tetrazine. However, a significant loss of diester tetrazine **46** occurs during recrystallisation.

The (bis)transesterification of diester dihydrotetrazine **45** with primary alcohols in the presence of aluminium ethoxide to give symmetrical diester dihydro-1,2,4,5-tetrazines has been reported at 39-85 % yield;[94] or with primary or secondary alcohols to obtain symmetrical and unsymmetrical diester dihydro-1,2,4,5-tetrazines at 21-44 % yield.[20] However, transesterification is not suitable for the late stage incorporation of diester dihydrotetrazine **45** onto the peptide backbone due to mechanistic considerations. The methanol generated *in situ* would need to be removed and/or a large excess of the corresponding alcohol would be required to push the equilibrium in favour of transesterification. Although both tactics were used for the bistransesterification of diester dihydrotetrazine **45** in the presence of aluminium ethoxide a near quantitative or quantitative yield was never reported in the literature.

The bisamidation of diester dihydrotetrazine **45** with primary or secondary amines to give symmetrical diamide dihydro-1,2,4,5-tetrazines has been reported at 79-98 % yield.[20] The near quantitative yields and absence of any mechanistic considerations make amidation with primary or secondary amines suitable for the late stage incorporation of diester dihydrotetrazine **45** onto the peptide backbone. Only a slight excess of the corresponding amine was reported in the literature for the bisamidation

of diester dihydrotetrazine **45** due to the increased nucleophilicity and decreased leaving group ability of amines relative to alcohols. However, a large excess of diester dihydrotetrazine **45** could be used to push the late stage incorporation of diester dihydrotetrazine **45** onto the peptide backbone if required. Therefore, the amidation of diester dihydrotetrazine **45** with primary or secondary amines was selected for the late stage incorporation of diester dihydrotetrazine **45** onto the peptide backbone.

3.3. Selection and Synthesis of Dienophiles

Strained cycloalkenes and cycloalkynes are highly reactive dienophiles in the iedDA reaction of 1,2,4,5-tetrazines as ring strain decreases the distortion energy required to reach the [4+2] cycloaddition transition state.[95-97] Also, numerous strained cycloalkenes and cycloalkynes which contain functional handles are readily available due to their extensive use in strain-promoted bioconjugation strategies.[69,75,98-100] Furthermore, these dienophiles are readily incorporated into N_{α}-protected lysine and are stable to the deprotection, coupling and cleavage cycles of SPPS.[101-103] Therefore, (*E*)-cycloalkene **49**, cycloalkyne **50** and (*Z*)-bicycloalkene **51** were selected as model dienophiles for the iedDA reactions of diester tetrazine **46** (Figure 4).

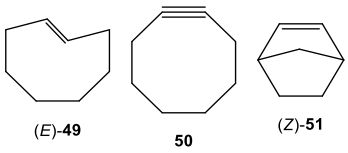
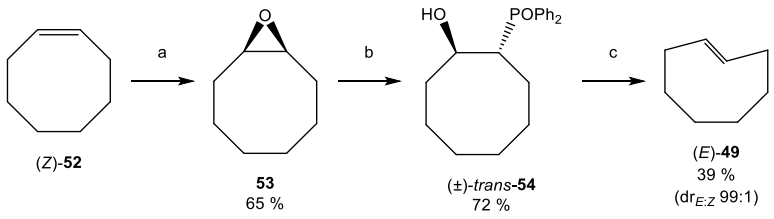


Figure 4. Dienophile selection for the iedDA reactions of diester tetrazine **46**.

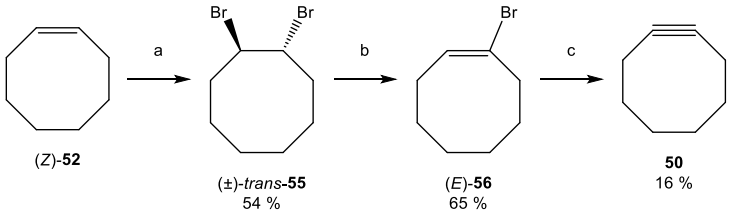
Many syntheses of (*E*)-cycloalkene **49** have been reported within the literature.[104-107] However, it was decided to follow a literature procedure which used phosphine oxides to facilitate the isomerisation of (*Z*)-alkenes as it was the most established non-photochemical synthesis of (*E*)-cycloalkene **49** within the literature.[108,109] The epoxidation of (*Z*)-cycloalkene **52** with 3-chloroperbenzoic acid (*m*CPBA) to give cycloalkane epoxide **53** was reported at 98 % yield. Successive ring opening of cycloalkane epoxide **53** by the nucleophilic addition of lithiodiphenylphosphane; and subsequent oxidation and protonation of the resulting lithium salt to give (±)-*trans*-

alcohol phosphine oxide cycloalkane **54** was reported at 77 % yield. The stereospecific base promoted elimination of sodium diphenylphosphinate from (±)-*trans*-alcohol phosphine oxide cycloalkane **54** to give (*E*)-cycloalkene **49** was reported at 58 % yield with less than 1 % (*Z*)-cycloalkene **52** impurity as determined by gas chromatography. Upon following the literature procedure (*E*)-cycloalkene **49** was isolated in 18 % overall yield with a 1 % (*Z*)-cycloalkene **52** impurity as determined by proton NMR (Scheme 18).



Scheme 18. Synthesis of (*E*)-cycloalkene **49**. Reagents and reaction conditions a) *m*CPBA (1 eq), DCM, rt, 5 h;[108] b) i) $LiPPh_2$ in THF (1.1 eq), THF, rt, 2 d, N_2; ii) AcOH (1.25 eq), H_2O_2 in H_2O, rt, 1 h;[108] c) NaH (1.2 eq), DMF, rt, 3 h, N_2.[108]

The established synthesis of cycloalkyne **50** was developed due to the inefficiencies of previous syntheses reported within the literature.[110-112] The electrophilic addition of bromine to (*Z*)-cycloalkene **52** and subsequent dehydrobromination of (±)-*trans*-dibromocycloalkane **55** with potassium *tert*-butoxide to give (*E*)-bromocycloalkene **56** was reported at 72 % yield (over two steps). Successive dehydrobromination of (*E*)-bromocycloalkene **56** with lithium diisopropylamide (LDA) to give cycloalkyne **50** was reported at 80-84 % yield. However, upon following the literature procedure cycloalkyne **50** was isolated in 6 % overall yield (Scheme 19). A significant loss of material occurred during the distillation of cycloalkyne **50** due to the small scale of the synthesis when compared with the literature.

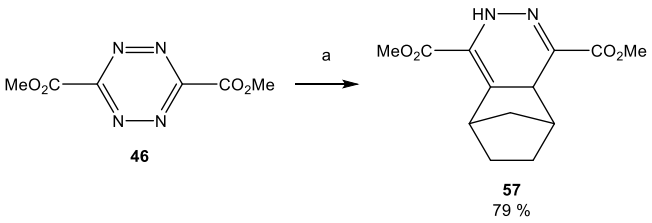


Scheme 19. Synthesis of cycloalkyne **50**. Reagents and reaction conditions a) Br_2 (1 eq), DCM, -40 °C, 30 min;[110] b) ^{t}BuOK in THF (1.5 eq), Et_2O, rt, 1 h, N_2;[110] c) LDA in hexanes and THF (0.5 eq), rt, 2.5 h, N_2.[110]

No synthesis was required for (*Z*)-bicycloalkene **51** as it was commercially available.

3.4. Inverse Electron Demand Diels-Alder Reactions

The first iedDA reaction to be screened was the iedDA reaction of diester tetrazine **46** with (*Z*)-bicycloalkene **51** as the synthesis of diester tricyclodihydropyridazine **57** had been previously reported within the literature.[113] The iedDA reaction of diester tetrazine **46** with (*Z*)-bicycloalkene **51** to give diester tricyclodihydropyridazine **57** was reported at 87 % yield in diethyl ether. Once the literature procedure had been verified the iedDA reaction of diester tetrazine **46** with (*Z*)-bicycloalkene **51** was repeated in dichloromethane (DCM) to give diester tricyclodihydropyridazine **57** in 79 % isolated yield (Scheme 20). Also, the concentration of diester tetrazine **46** had been decreased from 0.2 to 0.1 moldm^{-3} (M). Although the iedDA reaction with (*Z*)-bicycloalkene **51** had been left for 2 hours (h) it had gone to completion within a few minutes as indicated by the loss of red colour from diester tetrazine **46**.



Scheme 20. iedDA reaction of diester tetrazine **46** with (*Z*)-bicycloalkene **51**. Reagents and reaction conditions a) (*Z*)-bicycloalkene **51** (1.05 eq), DCM, rt, 2 h.

Alternative reaction conditions were used from those reported in the literature to mimic conditions commonly used in SPPS and therefore to be used for peptide macrocyclisation.[113] Although *N*,*N*-dimethylformamide (DMF) is a more widely used solvent in SPPS it was not selected for the solid-phase oxidation activation peptide macrocyclisation strategy due to its residual water content and the poor hydrolytic stability of diester tetrazine **46**.[114] During a qualitative hydrolytic stability study of a library of 1,2,4,5-tetrazines complete hydrolysis of diester tetrazine **46** was observed after 30 minutes (min) in DMF (Figure 5). However, complete hydrolysis of diester tetrazine **46** was not observed even after 2 days (d) in the analogous qualitative hydrolytic stability study of a library of 1,2,4,5-tetrazines in DCM.

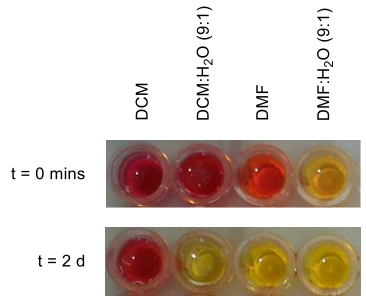
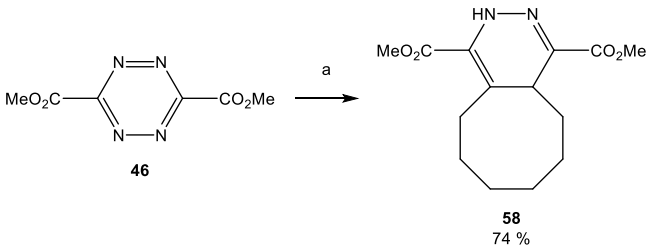


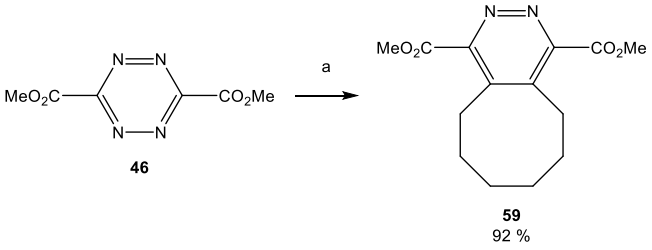
Figure 5. Qualitative hydrolytic stability study of 0.1 M diester tetrazine **46**. Complete hydrolysis of diester tetrazine **46** was indicated by a colour change from red to yellow.

The second iedDA reaction to be screened was the iedDA reaction of diester tetrazine **46** with (*E*)-cycloalkene **49** to give diester bicyclodihydropyridazine **58** (Scheme 21). With the alternative reaction conditions diester bicyclodihydropyridazine **58** was isolated in 74 % yield which was comparable to the yield achieved for the analogous (*Z*)-bicycloalkene **51** iedDA reaction. As expected, due to the increased reactivity of (*E*)-cycloalkene **49** over (*Z*)-bicycloalkene **51** in the iedDA reaction of 1,2,4,5-tetrazines, the iedDA reaction with (*E*)-cycloalkene **49** had gone to completion within a few minutes as indicated by the loss of red colour from diester tetrazine **46**.[95]



Scheme 21. iedDA reaction of diester tetrazine **46** with (*E*)-cycloalkene **49**. Reagents and reaction conditions a) (*E*)-cycloalkene **49** (1.05 eq), DCM, rt, 2 h.

The final iedDA reaction to be screened was the iedDA reaction of diester tetrazine **46** with cycloalkyne **50** to give diester bicyclopyridazine **59** (Scheme 22). With the alternative reaction conditions diester bicyclopyridazine **59** was isolated in 92 % yield which was a significant improvement over the analogous (*Z*)-bicycloalkene **51** and (*E*)-cycloalkene **49** iedDA reactions. Again, although the iedDA reaction with cycloalkyne **50** had been left for 2 h it had gone to completion within a few minutes as indicated by the loss of red colour from diester tetrazine **46**.



Scheme 22. iedDA reaction of diester tetrazine **46** with cycloalkyne **50**. Reagents and reaction conditions a) cycloalkyne **50** (1.05 eq), DCM, rt, 2 h.

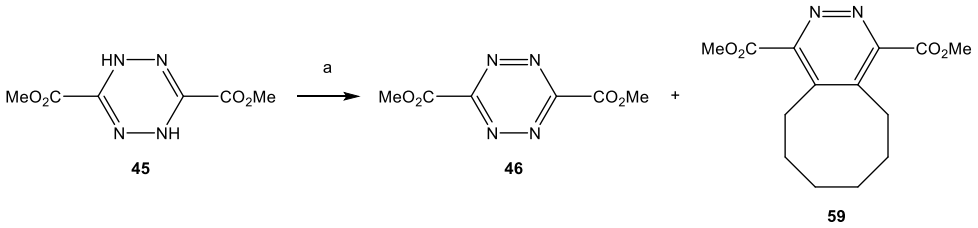
Cycloalkyne **50** was selected as the model dienophile for the proof of concept *in situ* oxidation and iedDA reaction of a dihydro-1,2,4,5-tetrazine due to its improved isolated yield over the analogous iedDA reactions of diester tetrazine **46** with (*Z*)-bicycloalkene **51** and (*E*)-cycloalkene **49**. Also, evidence of quantitative conversion to diester bicyclopyridazine **59** was obtained by proton NMR as no starting material, decomposition or side products were observed in the crude material. Furthermore, the direct formation of a diastereomerically pure pyridazine, when using an alkyne over an alkene dienophile, eliminates the oxidation of dihydropyridazine diastereomers and

therefore an additional level of complexity in the solid-phase oxidation activation peptide macrocyclisation strategy.

3.5. *In situ* Oxidation and Inverse Electron Demand Diels-Alder Reactions

As the oxidation of diester dihydrotetrazine **45** with isopentyl nitrite and the iedDA reaction of diester tetrazine **46** with cycloalkyne **50** were quantitative by proton NMR the *in situ* oxidation and iedDA reaction of diester dihydrotetrazine **45** with cycloalkyne **50** in the presence of isopentyl nitrite was optimised using proton NMR (Table 1). Initially 3 equivalents (eq) of isopentyl nitrite were used in the *in situ* oxidation and iedDA reaction of diester dihydrotetrazine **45** with cycloalkyne **50** to replicate the literature procedure for the oxidation of diester dihydrotetrazine **45** with isopentyl nitrite (Entry I).[80] Quantitative oxidation to diester tetrazine **46** was achieved but the subsequent iedDA reaction with cycloalkyne **50** to give diester bicyclopyridazine **59** occurred in 60 % proton NMR yield. Decomposition of cycloalkyne **50** by isopentyl nitrite was proposed to provide a competing pathway which limited the iedDA reaction of diester tetrazine **46**. The decomposition of cycloalkyne **50** by isopentyl nitrite was corroborated by conducting a stability study of cycloalkyne **50** towards isopentyl nitrite. Therefore, a second reaction was conducted with 1.05 eq of isopentyl nitrite to limit the decomposition of cycloalkyne **50** but with an insignificant effect on the yields of diester tetrazine **46** and diester bicyclopyridazine **59** (Entry II). However, when a third iedDA reaction of diester dihydrotetrazine **45** with cycloalkyne **50** was conducted with 10 eq of isopentyl nitrite there was a significant improvement in the yield achieved for diester bicyclopyridazine **59** (Entry III). When a final reaction was conducted with 25 eq of isopentyl nitrite the *in situ* oxidation and iedDA reaction of diester dihydrotetrazine **45** with cycloalkyne **50** to give diester bicyclopyridazine **59** was achieved in quantitative proton NMR yield (Entry IV). The oxidation of diester dihydrotetrazine **45** with isopentyl nitrite was proposed to have a higher rate constant than the degradation of cycloalkyne **50** by isopentyl nitrite but not corroborated by conducting kinetic studies of the oxidation of diester dihydrotetrazine **45** with isopentyl nitrite or the degradation of cycloalkyne **50** by isopentyl nitrite. Therefore, it was proposed that the *in situ* oxidation and iedDA of

diester dihydrotetrazine **45** with cycloalkyne **50** was favoured over the degradation of cycloalkyne **50** by isopentyl nitrite at increased concentrations of isopentyl nitrite.



Entry	Equivalents of Isopentyl Nitrite	^{1}H NMR Yield/%[a,b]	
		46	**59**
I	3	40	60
II	1.05	43	57
III	10	20	80
IV	25	0	Quantitative

Table 1. Optimisation of the *in situ* oxidation and iedDA reaction of diester dihydrotetrazine **45** with cycloalkyne **50** in the presence of isopentyl nitrite. Reagents and reaction conditions a) cycloalkyne **50** (1.05 eq), isopentyl nitrite, DCM, rt, 2 h. [a] Pseudoquantitative ^{1}H NMR yields were calculated from the ratio of diester tetrazine **46** to diester bicyclopyridazine **59** in the ^{1}H NMR spectrum. [b] Diester dihydrotetrazine **45** and cycloalkyne **50** were not observed.

3.6. Conclusions

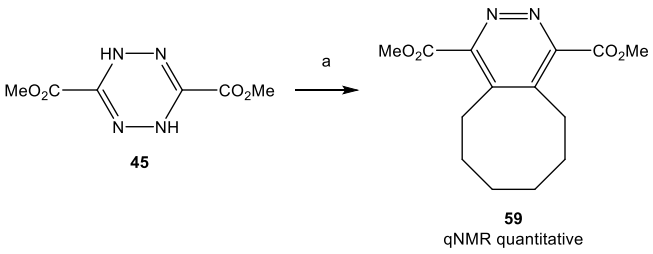
The aim of this chapter was to determine the viability of the solid-phase oxidation activation peptide macrocyclisation strategy by conducting a proof of concept *in situ* oxidation and iedDA reaction of a dihydro-1,2,4,5-tetrazine.

Diester dihydrotetrazine **45** was selected for the proof of concept *in situ* oxidation and iedDA reaction of a dihydro-1,2,4,5-tetrazine as its synthesis had been previously reported within the literature;[22] it can be readily oxidised to the reactive diester tetrazine **46** with isopentyl nitrite which is amenable to SPPS;[80] and the amidation of diester dihydrotetrazine **45** with primary or secondary amines can be used for the late stage incorporation of diester dihydrotetrazine **45** onto the peptide backbone.

(*E*)-Cycloalkene **49**, cycloalkyne **50** and (*Z*)-bicycloalkene **51** were selected as model dienophiles for the iedDA reactions of diester tetrazine **46** as strained cycloalkenes and cycloalkynes are highly reactive dienophiles in the iedDA reaction of 1,2,4,5-tetrazines;[95,96] numerous strained cycloalkenes and cycloalkynes which contain functional handles are available due to their extensive use in strain-promoted bioconjugation strategies;[69,75,98-100] and these dienophiles are readily incorporated into N_{α}-protected lysine and are stable to the deprotection, coupling and cleavage cycles of SPPS.[101-103]

Cycloalkyne **50** was selected as the model dienophile for the proof of concept *in situ* oxidation and iedDA reaction of a dihydro-1,2,4,5-tetrazine due to its improved isolated yield over the analogous iedDA reactions of diester tetrazine **46** with (*Z*)-bicycloalkene **51** and (*E*)-cycloalkene **49**.

The *in situ* oxidation and iedDA reaction of diester dihydrotetrazine **45** with cycloalkyne **50** in the presence of isopentyl nitrite to give diester bicyclopyridazine **59** was achieved in quantitative proton NMR yield (Scheme 23). Therefore, a successful proof of concept *in situ* oxidation and iedDA reaction of a dihydro-1,2,4,5-tetrazine was achieved. However, the *in situ* oxidation and iedDA reaction of diester dihydrotetrazine **45** with cycloalkyne **50** in the presence of isopentyl nitrite also highlighted the poor stability of a strained dienophile towards an oxidant.



Scheme 23. *In situ* oxidation and iedDA reaction of diester dihydrotetrazine **45** with cycloalkyne **50** in the presence of isopentyl nitrite. Reagents and reaction conditions a) cycloalkyne **50** (1.05 eq), isopentyl nitrite (25 eq), DCM, rt, 2 h.

Chapter 4. Optimisation in Solution-Phase

4.1. Aims

The aim of this chapter was to optimise the proof of concept *in situ* oxidation and iedDA reaction of a dihydro-1,2,4,5-tetrazine so that the optimised *in situ* oxidation and iedDA reaction of diester dihydrotetrazine **45** can be transferred to solid-phase. Two strategies were proposed to overcome the poor stability of a strained dienophile towards an oxidant and therefore optimise the proof of concept *in situ* oxidation and iedDA reaction of a dihydro-1,2,4,5-tetrazine.

Firstly, an alternative oxidant which does not facilitate decomposition of a strained dienophile can overcome the poor stability of a strained dienophile towards an oxidant. Therefore, a library of oxidants which are amenable to SPPS needed to be identified and their oxidation of diester dihydrotetrazine **45** screened before their use in an *in situ* oxidation and iedDA reaction of diester dihydrotetrazine **45**.

Secondly, an unstrained dienophile which is stable to decomposition by an oxidant can overcome the poor stability of a strained dienophile towards an oxidant. Therefore, a library of reactive unstrained dienophiles needed to be identified and synthesised. These dienophiles required a functional handle to enable amino acid derivatisation; and the resulting amino acids needed to be stable to the deprotection, coupling and cleavage cycles of SPPS.

The iedDA reactions of diester tetrazine **46** with the unstrained dienophiles needed to be screened before their use in an *in situ* oxidation and iedDA reaction of diester dihydrotetrazine **45**.

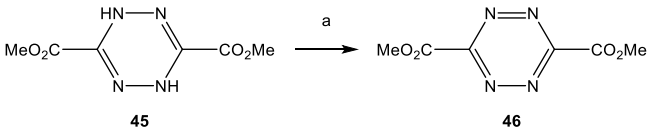
4.2. Oxidation of Dihydro-1,2,4,5-tetrazine

A quantitative analytic technique was required to enable an efficient and high-throughput investigation into the oxidation of diester dihydrotetrazine **45** which

negated the requirement to isolate diester tetrazine **46**. Quantitative nuclear magnetic resonance (qNMR) spectroscopy was selected for the investigation into the oxidation of diester dihydrotetrazine **45** as it fulfilled the requirements of the investigation but could also be used to monitor iedDA reactions of diester tetrazine **46** and investigate *in situ* oxidation and iedDA reactions of diester dihydrotetrazine **45**. 1,2,4,5-Tetramethylbenzene (durene) was selected as the qNMR internal standard as it was commercially available as a qNMR internal standard; and its Ar*H* and ArC*H*$_3$ peaks would not overlap with additional peaks in the proton NMR spectra and therefore prevent accurate calculation of qNMR yields. No optimisation was required to the open access proton NMR experimental parameters for qNMR as verified by authenticated samples of diester dihydrotetrazine **45** and diester tetrazine **46** in the presence of durene.

Oxidants were selected for the investigation into the oxidation of diester dihydrotetrazine **45** which are amenable to SPPS and had been previously reported within the literature for the oxidation of a dihydro-1,2,4,5-tetrazine or a dihydropyridazine (Table 2).[115-122] During the investigation into the oxidation of diester dihydrotetrazine **45** 3 eq of oxidant were used to replicate the literature procedure for the oxidation of diester dihydrotetrazine **45** with isopentyl nitrite.[80] Unexpectedly, the oxidation of diester dihydrotetrazine **45** with isopentyl nitrite to give diester tetrazine **46** was achieved in 65 % qNMR yield (Entry I). However, no starting material, decomposition or side products were observed in the proton NMR spectrum. Intermolecular proton spin energy transfer between diester tetrazine **46** and isopentyl nitrite was proposed but discredited by an authenticated sample of diester tetrazine **46** and isopentyl nitrite in the presence of durene. The analogous 2,3-dichloro-5,6-dicyano-*p*-benzoquinone (DDQ), [bis(trifluoroacetoxy)iodo]benzene (BTI) and 4-phenyl-1,2,4-triazoline-3,5-dione (PTAD) oxidations were achieved in quantitative qNMR yield whilst partial oxidation was achieved in the diethyl azodicarboxylate (DEAD) and chloranil reactions (Entries II-IV and VI-VII). The qNMR results of the oxidation of diester dihydrotetrazine **45** with DDQ, BTI and PTAD were corroborated by authenticated samples of diester tetrazine **46** and the respective oxidant in the presence of durene. The oxidation of diester dihydrotetrazine **45** with ammonium

cerium(IV) nitrate (CAN) and trimethylphenylammonium tribromide (TPAT) to give diester tetrazine **46** was observed in 88 and 7 % qNMR yield respectively (Entries V and VIII). Decomposition of diester tetrazine **46** by CAN and TPAT was proposed but not corroborated by conducting stability studies of diester tetrazine **46** towards CAN and TPAT.



45 **46**

Entry	Oxidant	qNMR Yield/%	
		45	**46**
I	Isopentyl nitrite	0	65
II	DDQ	0	Quantitative
III	DEAD	89	11
IV	BTI	0	Quantitative
V	CAN	0	88
VI	Chloranil	94	6
VII	PTAD	0	Quantitative
VIII	TPAT	75	7

Table 2. Investigation into the oxidation of diester dihydrotetrazine **45**. Reagents and reaction conditions a) durene (0.5 eq), oxidant (3 eq), DCM-d_2, 25 °C, 2 h.

DDQ, BTI and PTAD were selected as alternative oxidants to optimise the proof of concept *in situ* oxidation and iedDA reaction of a dihydro-1,2,4,5-tetrazine due to their corroborated quantitative qNMR yields in the oxidation of diester dihydrotetrazine **45**.

4.3. Selection and Synthesis of Dienophiles

endo- and *exo*-BCN-OH **60** were selected as dienophiles for the iedDA reactions of diester tetrazine **46** as they are highly reactive in the iedDA reaction of 1,2,4,5-tetrazines; are readily incorporated into Fmoc-Lys-OH hydrochloride; and are stable to the deprotection, coupling and cleavage cycles of SPPS (Figure 6).[100,103,123] Also, the iedDA reaction of diester tetrazine **46** with *endo*- and *exo*-BCN-OH **60** will give a single regioisomerically pure pyridazine due to the C_s symmetry of *endo*- and *exo*-BCN-OH **60**. Unstrained cycloalkenes and terminal alkenes are reactive dienophiles

in the iedDA reaction of 1,2,4,5-tetrazines.[95,96] Therefore, (±)-(*Z*)-cycloalkene alcohol **61**, allyl amide **62** and allyl ester **63** were selected as dienophiles for the iedDA reactions of diester tetrazine **46**. (±)-(*Z*)-cycloalkene alcohol **61** contains a functional handle for amino acid derivatisation whilst allyl amide **62** and allyl ester **63** model $N_{\beta/\gamma}$-allyl amine derivatised Fmoc-Asp-OH and Fmoc-Glu-OH; and Fmoc-Asp(OAll)-OH and Fmoc-Glu(OAll)-OH respectively.

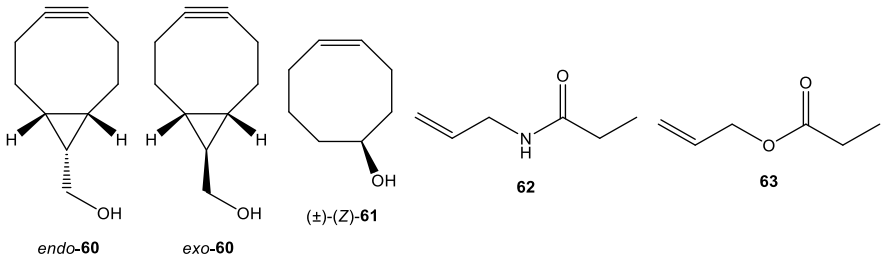
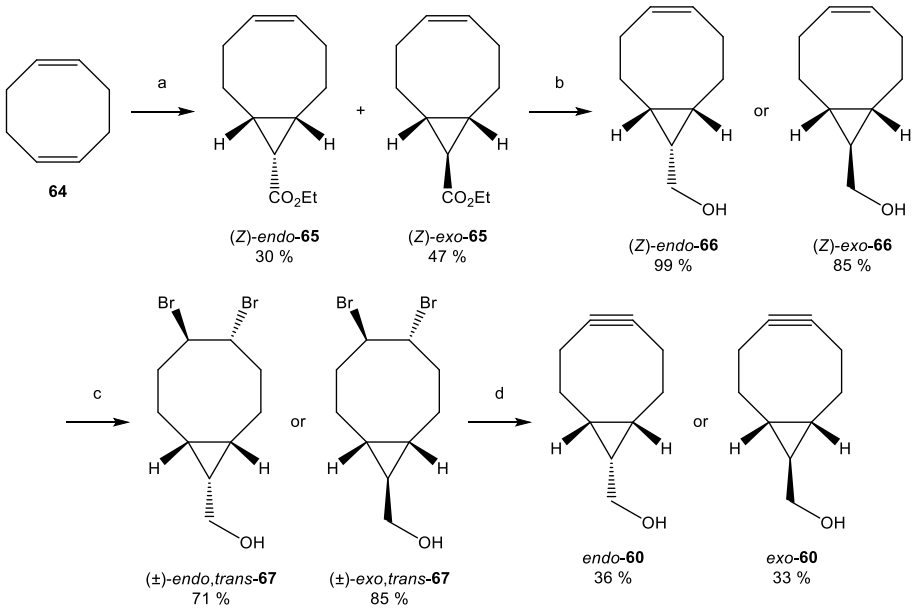


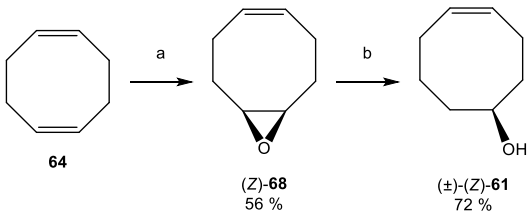
Figure 6. Dienophile selection for the iedDA reactions of diester tetrazine **46**.

The established synthesis of *endo*- and *exo*-BCN-OH **60** was developed to provide a highly reactive, novel and readily accessible strained cycloalkyne for use in strain-promoted bioconjugation strategies.[100] The cyclopropanation of cyclodiene **64** with ethyl diazoacetate in the presence of rhodium(II) acetate dimer to give (*Z*)-*endo*- and (*Z*)-*exo*-bicycloalkene ester **65** was reported at 25 and 51 % yield respectively after diasteromeric separation by flash column chromatography. Successive reduction of (*Z*)-*endo*-bicycloalkene ester **65** with lithium aluminium hydride was reported to give (*Z*)-*endo*-bicycloalkene alcohol **66**. The electrophilic addition of bromine to (*Z*)-*endo*-bicycloalkene alcohol **66** and subsequent bisdehydrobromination of (±)-*endo*,*trans*-dibromobicycloalkane alcohol **67** with potassium *tert*-butoxide to give *endo*-BCN-OH **60** was reported at 61 % yield (over three steps). The analogous reduction, electrophilic addition and bisdehydrobromination of (*Z*)-*exo*-bicycloalkene ester **65** to give *exo*-BCN-OH **60** was reported at 53 % yield. Upon following the literature procedure with additional purifications *endo*- and *exo*-BCN-OH **60** were isolated in 8 and 11 % overall yield respectively (Scheme 24).



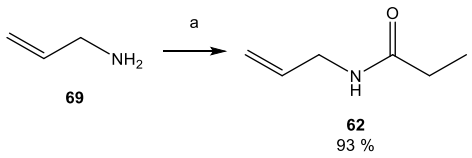
Scheme 24. Syntheses of *endo*- and *exo*-BCN-OH **60**. Reagents and reaction conditions a) $Rh_2(OAc)_4$ (5 mol%), ethyl diazoacetate (0.125 eq), DCM, rt, 40 h, N_2;[100] b) $LiAlH_4$ (0.9 eq), Et_2O, rt, 30 min, N_2;[100] c) Br_2 (1.1 eq), DCM, 0 °C, 30 min;[100] d) ^{t}BuOK in THF(3.3 eq), THF, reflux, 2 h, N_2.[100]

The established synthesis of (±)-(*Z*)-cycloalkene alcohol **61** was developed to enable the synthesis of 9-oxabicyclo[6.1.0]non-3-yne.[124,125] The epoxidation of cyclodiene **64** with *m*CPBA to give (*Z*)-cycloalkene epoxide **68** was reported at 66 % yield. Successive ring opening of (*Z*)-cycloalkene epoxide **68** by the nucleophilic addition of lithium aluminium hydride and subsequent protonation of the resulting lithium salt to give (±)-(*Z*)-cycloalkene alcohol **61** was reported at quantitative yield. However, upon following the literature procedure (±)-(*Z*)-cycloalkene alcohol **61** was isolated in 40 % overall yield (Scheme 25). No optimisation was conducted to the literature procedure as sufficient material was obtained for the iedDA reaction of diester tetrazine **46**.



Scheme 25. Synthesis of (±)-(*Z*)-cycloalkene alcohol **61**. Reagents and reaction conditions a) *m*CPBA (1 eq), $CHCl_3$, rt, ~18 h;[125] b) $LiAlH_4$ in THF (0.5 eq), THF, rt, ~18 h, N_2.[125]

The nucleophilic substitution of propionyl chloride with allyl amine **69** in the presence of triethylamine to give allyl amide **62** was achieved in 93 % isolated yield (Scheme 26).



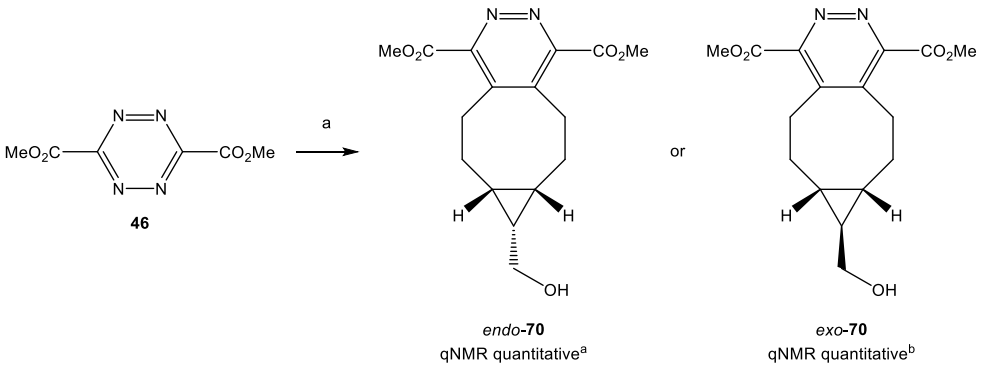
69 **62** 93 %

Scheme 26. Synthesis of allyl amide **62**. Reagents and reaction conditions a) EtCOCl (1 eq), Et_3N (1.2 eq), DCM, rt, 2 h.

No synthesis was required for allyl ester **63** as it was commercially available.

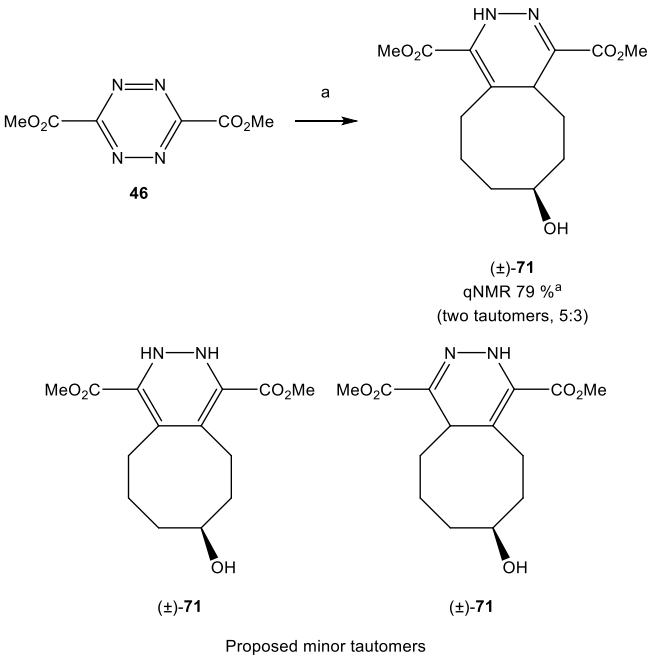
4.4. Inverse Electron Demand Diels-Alder Reactions

The first iedDA reactions to be screened were the iedDA reactions of diester tetrazine **46** with *endo*- and *exo*-BCN-OH **60** (Scheme 27). The iedDA reactions of diester tetrazine **46** with *endo*- and *exo*-BCN-OH **60** to give *endo*- and *exo*-diester tricyclopyridazine alcohol **70** were achieved in quantitative qNMR yield. As expected, no observable differences were encountered between the iedDA reactions of diester tetrazine **46** with *endo*- and *exo*-BCN-OH **60**.[100] Although the iedDA reactions with *endo*- and *exo*-BCN-OH **60** had been left for 2 h they had gone to completion within a few minutes as indicated by the loss of red colour from diester tetrazine **46**. To corroborate the qNMR results *endo*- and *exo*-diester tricyclopyridazine alcohol **70** were isolated in 85 and 89 % yield respectively.



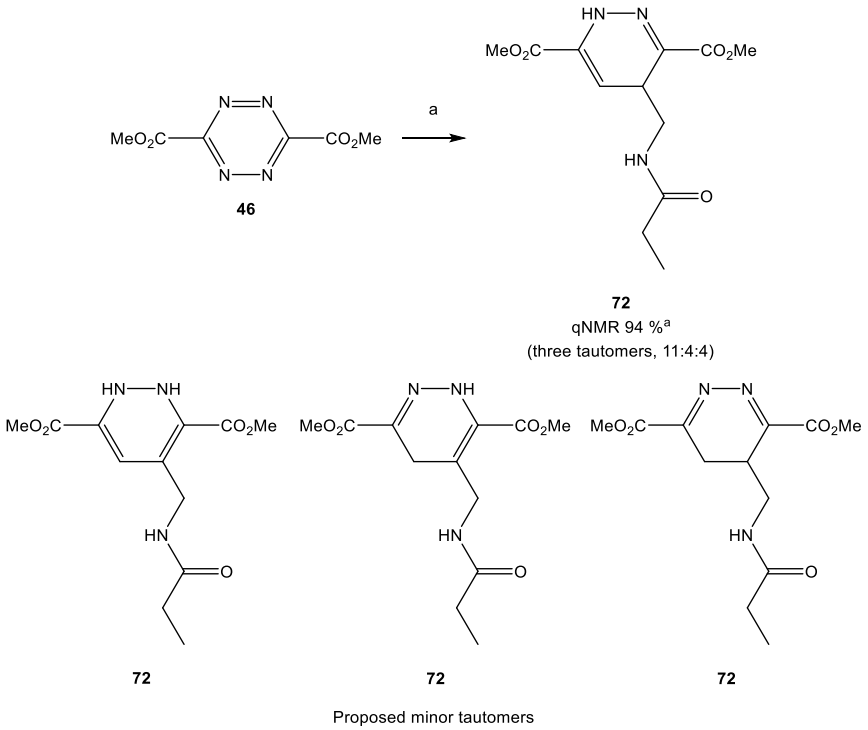
Scheme 27. iedDA reactions of diester tetrazine **46** with *endo*- and *exo*-BCN-OH **60**. Reagents and reaction conditions a) durene (0.5 eq), *endo*- or *exo*-BCN-OH **60** (1.05 eq), DCM-d_2, 25 °C, 2 h. [a] *endo*-diester tricyclopyridazine alcohol **70** was isolated in 85 % yield. [b] *exo*-diester tricyclopyridazine alcohol **70** was isolated in 89 %.

The second iedDA reaction to be screened was the iedDA reaction of diester tetrazine **46** with (±)-(*Z*)-cycloalkene alcohol **61** (Scheme 28). The iedDA reaction of diester tetrazine **46** with (±)-(*Z*)-cycloalkene alcohol **61** to give (±)-diester bicyclodihydropyridazine alcohol **71** as a mixture of two tautomers was achieved in 79 % qNMR yield. The major tautomer was identified by characteristic RN*H*N and R_3C*H* peaks in the proton NMR spectrum. However, several diastereomers and regioisomers of the major tautomer exist which are not distinguishable by one dimensional proton NMR. A minor tautomer was identified due to the presence of additional RN*H*N and CO_2C*H*$_3$ peaks in the proton NMR spectrum but could not be assigned without characteristic peaks. Although the iedDA reaction with (±)-(*Z*)-cycloalkene alcohol **61** had been left for 2 h it had gone to completion within 1 h as indicated by the loss of red colour from diester tetrazine **46**. No attempts were made to corroborate the qNMR result by isolating (±)-diester bicyclodihydropyridazine alcohol **71** due to the quantitative qNMR yield achieved in the analogous *endo*- and *exo*-BCN-OH **60** iedDA reactions.



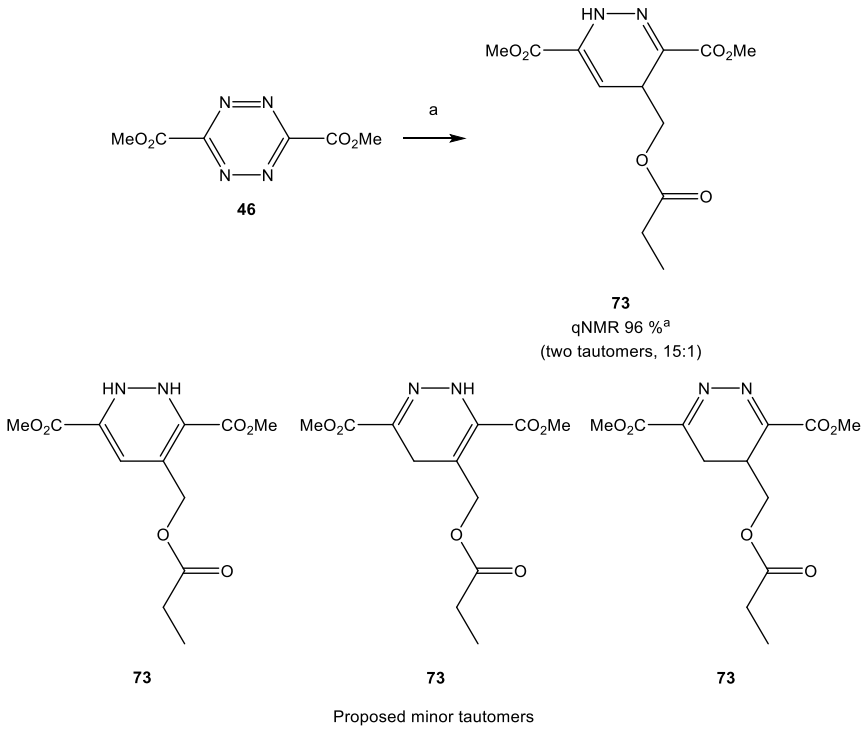
Scheme 28. iedDA reaction of diester tetrazine **46** with (±)-(*Z*)-cycloalkene alcohol **61**. Reagents and reaction conditions a) durene (0.5 eq), (±)-(*Z*)-cycloalkene alcohol **61** (1.05 eq), DCM-d_2, 25 °C, 2 h. [a] Not corroborated by the isolation of (±)-diester bicyclodihydropyridazine alcohol **71**.

The third iedDA reaction to be screened was the iedDA reaction of diester tetrazine **46** with allyl amide **62** (Scheme 29). The iedDA reaction of diester tetrazine **46** with allyl amide **62** to give diester dihydropyridazine amide **72** as a mixture of three tautomers was achieved in 94 % qNMR yield. The major tautomer was assigned by characteristic RN*H*N, RC*H*R and R_3C*H* peaks in the proton NMR spectrum. Two minor tautomers were identified due to the presence of additional CO_2CH_3, NHCOCH_2R and RCH_3 peaks in the proton NMR spectrum but could not be assigned without characteristic peaks. Although the iedDA reaction with allyl amide **60** had been left for 2 h it had gone to completion within 1.5 h as indicated by the loss of red colour from diester tetrazine **46**. All attempts to isolate diester dihydropyridazine amide **72** were unsuccessful and therefore the qNMR result was not corroborated by the isolation of diester dihydropyridazine amide **72**.



Scheme 29. iedDA reaction of diester tetrazine **46** with allyl amide **62**. Reagents and reaction conditions a) durene (0.5 eq), allyl amide **62** (1.05 eq), DCM-d_2, 25 °C, 2 h. [a] Not corroborated by the isolation of diester dihydropyridazine amide **72**.

The final iedDA reaction to be screened was the iedDA reaction of diester tetrazine **46** with allyl ester **63** (Scheme 30). The iedDA reaction of diester tetrazine **46** with allyl ester **63** to give diester dihydropyridazine ester **73** as a mixture of two tautomers was achieved in 96 % qNMR yield. The major tautomer was assigned by characteristic RN*H*N, RC*H*R and R_3C*H* peaks in the proton NMR spectrum. A minor tautomer was identified due to the presence of additional CO_2CH_3, OCOCH_2R and RCH_3 peaks in the proton NMR spectrum but could not be assigned without characteristic peaks. As expected, due to the increased electronegativity of oxygen over nitrogen and the inductive effect, the iedDA reaction of diester tetrazine **46** with allyl ester **63** exhibited a slower rate of reaction than the analogous allyl amide **62** iedDA reaction. To corroborate the qNMR result diester dihydropyridazine ester **73** was isolated in 84 % yield.

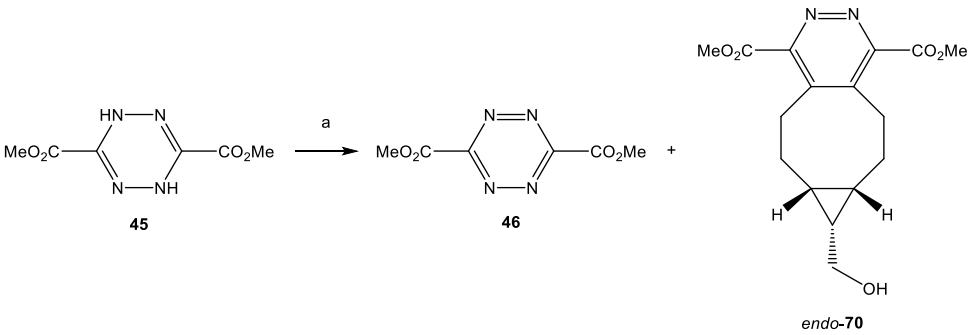


Scheme 30. iedDA reaction of diester tetrazine **46** with allyl ester **63**. Reagents and reaction conditions a) durene (0.5 eq), allyl ester **63** (1.05 eq), DCM-d_2, 25 °C, 2 h. [a] Diester dihydropyridazine ester **73** was isolated in 84 %.

endo-BCN-OH **60** and allyl ester **63** were selected as dienophiles to optimise the proof of concept *in situ* oxidation and iedDA reaction of a dihydro-1,2,4,5-tetrazine due to their corroborated quantitative and near quantitative qNMR yield respectively in the iedDA reactions of diester tetrazine **46**. *endo*-BCN-OH **60** was selected as a dienophile to optimise the proof of concept *in situ* oxidation and iedDA reaction of a dihydro-1,2,4,5-tetrazine over *exo*-BCN-OH **60** as this diastereomer was commercially available. Although a near quantitative qNMR yield had been achieved for the iedDA reaction of diester tetrazine **46** with allyl amide **62** this result was not corroborated by the isolation of diester dihydropyridazine amide **72**. Therefore, allyl amide **62** was not selected as a dienophile to optimise the proof of concept *in situ* oxidation and iedDA reaction of a dihydro-1,2,4,5-tetrazine.

4.5. *In situ* Oxidation and Inverse Electron Demand Diels-Alder Reactions

The first *in situ* oxidation and iedDA reaction to be investigated was the *in situ* oxidation and iedDA reaction of diester dihydrotetrazine **45** with *endo*-BCN-OH **60** (Table 3). The *in situ* oxidation and iedDA reaction of diester dihydrotetrazine **45** with *endo*-BCN-OH **60** in the presence of DDQ to give *endo*-diester tricyclopyridazine alcohol **70** was achieved in 97 % qNMR yield (Entry I). However, diester dihydrotetrazine **45**, diester tetrazine **46** and *endo*-diester tricyclopyridazine alcohol **70** were not observed by proton NMR in the analogous BTI *in situ* oxidation and iedDA reaction (Entry II). Decomposition of *endo*-diester tricyclopyridazine alcohol **70** by BTI was proposed but not corroborated by conducting a stability study of *endo*-diester tricyclopyridazine alcohol **70** towards BTI. In the presence of PTAD quantitative oxidation to diester tetrazine **46** was observed but the subsequent iedDA reaction with *endo*-BCN-OH **60** occurred in 61 % qNMR yield (Entry III). Decomposition of *endo*-BCN-OH **60** by PTAD provided a competing pathway which limited the iedDA reaction of diester tetrazine **46** with *endo*-BCN-OH **60**. No optimisation was conducted to the *in situ* oxidation and iedDA reaction of diester dihydrotetrazine **45** with *endo*-BCN-OH **60** in the presence of PTAD due to the near quantitative qNMR yield achieved in the analogous DDQ *in situ* oxidation and iedDA reaction.

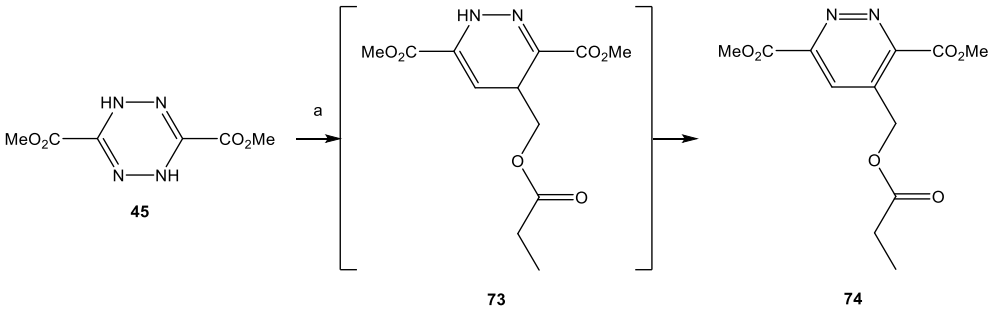


Entry	Oxidant	qNMR Yield/%[a]	
		46	**70**
I	DDQ	0	97
II	BTI	0	0
III[b]	PTAD	38	61

Table 3. Investigation into the *in situ* oxidation and iedDA reaction of diester dihydrotetrazine **45** with *endo*-BCN-OH **60**. Reagents and reaction conditions a) durene (0.5 eq), *endo*-BCN-OH **60** (1.05 eq), oxidant (3 eq), DCM-d_2, 25 °C, 2 h. [a] Diester dihydrotetrazine **45** was not observed. [b] *endo*-BCN-OH **60** was not observed.

The final *in situ* oxidation and iedDA reaction to be investigated was the *in situ* oxidation and iedDA reaction of diester dihydrotetrazine **45** with allyl ester **63** (Table 4). The *in situ* oxidation and iedDA reaction of diester dihydrotetrazine **45** with allyl ester **63** in the presence of DDQ and subsequent oxidation of diester dihydropyridazine ester **73** to give diester pyridazine ester **74** was achieved in quantitative qNMR yield (Entry I). To corroborate the qNMR result diester pyridazine ester **74** was isolated in 97 % yield. Diester dihydrotetrazine **45**, diester tetrazine **46**, diester dihydropyridazine ester **73** and diester pyridazine ester **74** were not observed by proton NMR in the analogous BTI *in situ* oxidation and iedDA reaction (Entry II). Decomposition of diester dihydropyridazine ester **73** and/or diester pyridazine ester **74** by BTI was proposed but not corroborated by conducting stability studies of diester dihydropyridazine ester **73** and diester pyridazine ester **74** towards BTI. The *in situ* oxidation and iedDA reaction of diester dihydrotetrazine **45** with ally ester **63** in the presence of PTAD to give diester pyridazine ester **74** occurred in 31 % qNMR yield (Entry III). However, diester dihydrotetrazine **45**, diester tetrazine **46**, and diester dihydropyridazine ester **73** were not observed by proton NMR. Therefore, decomposition of diester pyridazine ester **74** by PTAD was proposed but with a slower

rate of decomposition than the proposed decomposition of diester dihydropyridazine ester **73** and/or diester pyridazine ester **74** by BTI.



Entry	Oxidant	qNMR Yield/%[a]
		74
I	DDQ	Quantitative[b]
II	BTI	0
III	PTAD	31

Table 4. Investigation into the *in situ* oxidation and iedDA reaction of diester dihydrotetrazine **45** with allyl ester **63**. Reagents and reaction conditions a) durene (0.5 eq), allyl ester **63** (1.05 eq), oxidant (3 eq), DCM-d_2, 25 °C, 2 h. [a] Diester dihydrotetrazine **45**, diester tetrazine **46** and diester dihydropyridazine ester **73** were not observed. [b] Diester pyridazine ester **74** was isolated in 97 % yield.

4.6. Conclusions

The aim of this chapter was to optimise the proof of concept *in situ* oxidation and iedDA reaction of a dihydro-1,2,4,5-tetrazine so that the optimised *in situ* oxidation and iedDA reaction of diester dihydrotetrazine **45** can be transferred to solid-phase. Two strategies were proposed to overcome the poor stability of a strained dienophile towards an oxidant and therefore optimise the proof of concept *in situ* oxidation and iedDA reaction of a dihydro-1,2,4,5-tetrazine.

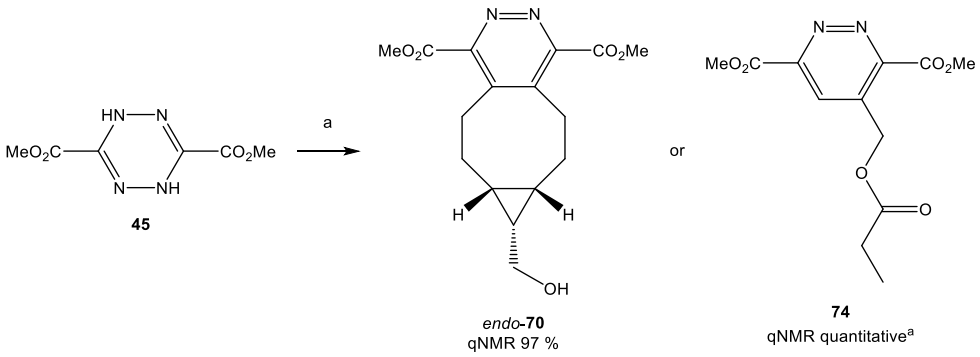
Firstly, an alternative oxidant which does not facilitate decomposition of a strained dienophile can overcome the poor stability of a strained dienophile towards an oxidant. Oxidants were selected for the investigation into the oxidation of diester dihydrotetrazine **45** which are amenable to SPPS and had been previously reported within the literature for the oxidation of a dihydro-1,2,4,5-tetrazine or a

dihydropyridazine.[115-122] DDQ, BTI and PTAD were selected as alternative oxidants to optimise the proof of concept *in situ* oxidation and iedDA reaction of a dihydro-1,2,4,5-tetrazine due to their corroborated quantitative qNMR yields in the oxidation of diester dihydrotetrazine **45**.

Secondly, an unstrained dienophile which is stable to decomposition by an oxidant can overcome the poor stability of a strained dienophile towards an oxidant. (±)-(*Z*)-cycloalkene alcohol **61**, allyl amide **62** and allyl ester **63** were selected as dienophiles for the iedDA reaction of diester tetrazine **46** as unstrained cycloalkenes and terminal alkenes are reactive dienophiles in the iedDA reaction of 1,2,4,5-tetrazines.[95,96] (±)-(*Z*)-cycloalkene alcohol **61** contains a functional handle for amino acid derivatisation whilst allyl amide **62** and allyl ester **63** model $N_{\beta/\gamma}$-allyl amine derivatised Fmoc-Asp-OH and Fmoc-Glu-OH; and Fmoc-Asp(OAll)-OH and Fmoc-Glu(OAll)-OH respectively.

endo-BCN-OH **60** and allyl ester **63** were selected as dienophiles to optimise the proof of concept *in situ* oxidation and iedDA reaction of a dihydro-1,2,4,5-tetrazine due to their corroborated quantitative and near quantitative qNMR yield respectively in the iedDA reactions of diester tetrazine **46**. *endo*-BCN-OH **60** was selected as a dienophile to optimise the proof of concept *in situ* oxidation and iedDA reaction of a dihydro-1,2,4,5-tetrazine over *exo*-BCN-OH **60** as this diastereomer was commercially available.

The *in situ* oxidation and iedDA reactions of diester dihydrotetrazine **45** with *endo*-BCN-OH **60** and allyl ester **63** in the presence of DDQ to give *endo*-diester tricyclopyridazine alcohol **70** and diester pyridazine ester **74** were achieved in 97 % and quantitative qNMR yield respectively (Scheme 31). Therefore, the proof of concept *in situ* oxidation and iedDA reaction of a dihydro-1,2,4,5-tetrazine was successfully optimised in the presence of an alternative oxidant and with an unstrained dienophile in the presence of the alternative oxidant.

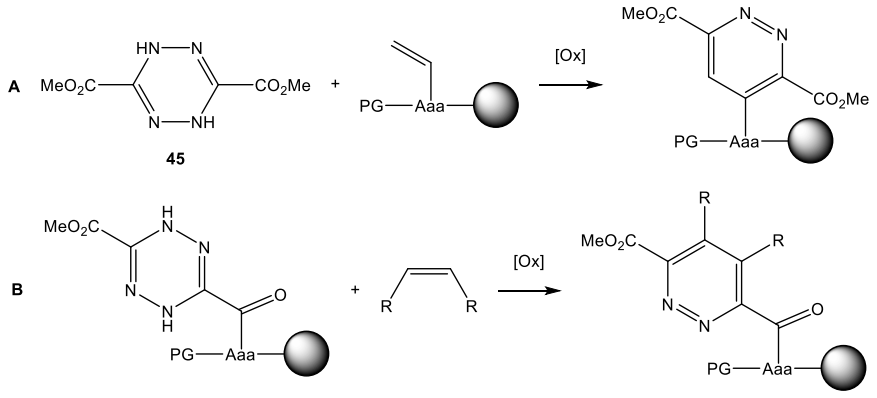


Scheme 31. *In situ* oxidation and iedDA reactions of diester dihydrotetrazine **45** with *endo*-BCN-OH **60** and allyl ester **63** in the presence of DDQ. Reagents and reaction conditions a) durene (0.5 eq), *endo*-BCN-OH **60** or allyl ester **63** (1.05 eq), DDQ (3 eq), DCM-d_2, 25 °C, 2 h. [a] Diester pyridazine ester **74** was isolated in 97 % yield.

Chapter 5. Transfer to Solid-Phase

5.1. Aims

The aims of this chapter were to transfer the optimised *in situ* oxidation and iedDA reactions of diester dihydrotetrazine **45** to solid-phase and to verify the *in situ* oxidation and iedDA reactions of resin-bound diester dihydrotetrazine derivatised amino acids in the presence of DDQ so that peptide macrocyclisations can be conducted on solid-phase (Scheme 32A and 32B). However, to successfully transfer the optimised *in situ* oxidation and iedDA reactions of diester dihydrotetrazine **45** to solid-phase and to verify the *in situ* oxidation and iedDA reactions of resin-bound diester dihydrotetrazine derivatised amino acids in the presence of DDQ several prerequisites needed to be achieved.



Scheme 32A. *In situ* oxidation and iedDA reaction of diester dihydrotetrazine **45** transferred to solid-phase. **Scheme 32B.** *In situ* oxidation and iedDA reaction of resin-bound diester dihydrotetrazine derivatised amino acid.

Firstly, the stability of resin-bound amino acids towards DDQ needed to be determined before transferring the optimised *in situ* oxidation and iedDA reactions of diester dihydrotetrazine **45** to solid-phase and verifying the *in situ* oxidation and iedDA reactions of resin-bound diester dihydrotetrazine derivatised amino acids in the presence of DDQ.

Secondly, Fmoc-Lys(*endo*-BCN)-OH **75** needed to be synthesised and the *in situ* oxidation and iedDA reactions of diester dihydrotetrazine **45** with resin-bound dienophiles in the presence of DDQ needed to be conducted to enable the optimised *in situ* oxidation and iedDA reactions of diester dihydrotetrazine **45** to be transferred to solid-phase.

Thirdly, resin-bound diester dihydrotetrazine derivatised amino acids needed to be synthesised to verify the late stage incorporation of diester dihydrotetrazine **45** onto the peptide backbone and the *in situ* oxidation and iedDA reactions of resin-bound diester dihydrotetrazine derivatised amino acids in the presence of DDQ.

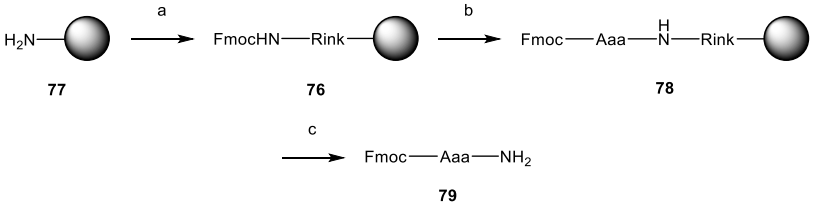
Finally, the *in situ* oxidation and iedDA reactions of resin-bound diester dihydrotetrazine derivatised amino acids with *endo*-BCN-OH **60** and allyl ester **63** in the presence of DDQ needed to be conducted to verify the *in situ* oxidation and iedDA reactions of resin-bound diester dihydrotetrazine derivatised amino acids in the presence of DDQ.

5.2. Stability of Resin-Bound Amino Acids towards Oxidant

An analytical technique was required to enable an efficient and high-throughput investigation into the stability of resin-bound amino acids towards DDQ. Analytical high-performance liquid chromatography (HPLC) was selected in combination with low-resolution mass spectroscopy (LRMS) for the investigation into the stability of resin-bound amino acids towards DDQ as these are commonly used analytical techniques to characterise peptides.

Commercially available N_{α}-9-fluorenylmethoxycarbonyl (Fmoc) protected standard proteinogenic amino acids were selected for the investigation into the stability of resin-bound amino acids towards DDQ as these are commonly used amino acids in SPPS and therefore to be used for peptide macrocyclisation (Table 5). However, any standard proteinogenic amino acids without a reactive functional group in their side chain were not included in the investigation into the stability of resin-bound amino acids towards

DDQ. All the standard proteinogenic amino acids with a reactive functional group in their side chain were protected with trifluoroacetic acid (TFA)-labile protecting groups which are orthogonal to the Fmoc protecting group, except Fmoc-Met-OH, to enable global deprotection of the macrocyclic peptide during linker cleavage. Rink amide polystyrene resin **76** was selected for the investigation into the stability of resin-bound amino acids towards DDQ as this resin was commercially available at various loading values and could therefore be used for both linear and macrocyclic peptide synthesis.



Entry	Fmoc-Aaa-OH	HPLC[b]	
		79[a]	Purity/%
I	Fmoc-Arg(Pbf)-OH	Fmoc-Arg-NH_2	100
II	Fmoc-Asn(Trt)-OH	Fmoc-Asn-NH_2	100
III	Fmoc-Asp(O^{t}Bu)-OH	Fmoc-Asp-NH_2	100
IV	Fmoc-Cys(Trt)-OH	Fmoc-Cys-NH_2	100
V	Fmoc-Gln(Trt)-OH	Fmoc-Gln-NH_2	100
VI	Fmoc-Glu(O^{t}Bu)-OH	Fmoc-Glu-NH_2	100
VII	Fmoc-His(Trt)-OH	Fmoc-His-NH_2	100
VIII	Fmoc-Lys(Boc)-OH	Fmoc-Lys-NH_2	100
IX	Fmoc-Met-OH	Fmoc-Met-NH_2	96
X	Fmoc-Ser(^{t}Bu)-OH	Fmoc-Ser-NH_2	100
XI	Fmoc-Thr(^{t}Bu)-OH	Fmoc-Thr-NH_2	100
XII	Fmoc-Trp(Boc)-OH	Fmoc-Trp-NH_2	100
XIII	Fmoc-Tyr(^{t}Bu)-OH	Fmoc-Tyr-NH_2	100

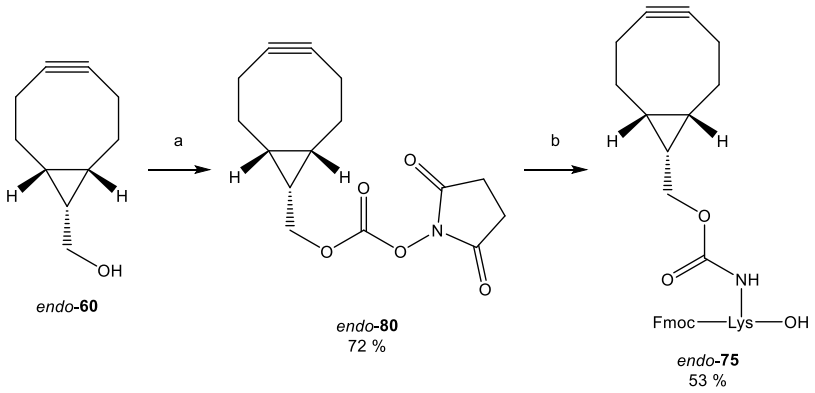
Table 5. Investigation into the stability of resin-bound amino acids towards DDQ. Reagents and reaction conditions a) Rink amide linker (3 eq), oxyma (3 eq), DIC (3 eq), DMF, rt, 45 min; b) i) piperidine in DMF, rt, 5 min; ii) Fmoc-Aaa-OH (3eq), oxyma (3 eq), DIC (3 eq), DMF, rt, 45 min; c) i) DDQ (3 eq), DCM, rt, 8 h; ii) TFA:TIS:DCM (90:5:5), rt, 1 h. [a] Identified by LRMS. [b] ELSD.

The resin functionalisation of amino polystyrene resin **77** with 4-[(2,4-dimethoxyphenyl)(Fmoc-amino)methyl]phenoxyacetic acid (Rink amide linker) in the presence of ethyl (hydroxyimino)cyanoacetate (oxyma) and *N*,*N*`-diisopropylcarbodiimide (DIC) gave Rink amide polystyrene resin **76** (Table 5).

Successive Fmoc deprotection of Rink amide polystyrene resin **76** with piperidine and subsequent amino acid coupling of Fmoc-Aaa-OH in the presence of oxyma and DIC gave resin-bound Fmoc-Aaa-OH **78**. Exposure of resin-bound Fmoc-Aaa-OH **78** to DDQ and subsequent linker cleavage with TFA:triisopropylsilane (TIS):DCM gave Fmoc-Aaa-NH_2 **79**. During the investigation into the stability of resin-bound amino acids towards DDQ 3 eq of oxidant were used to mimic the reaction conditions to be used for peptide macrocyclisation. The resin-bound Fmoc-Aaa-OH **78** was exposed to DDQ for 8 h as the optimised *in situ* oxidation and iedDA reactions of diester dihydrotetrazine **45** had gone to completion within 2 h. Therefore, the stability of resin-bound Fmoc-Aaa-OH **78** towards DDQ was tested beyond the exposure limits expected for peptide macrocyclisation. No decomposition, deprotection or oxidation of resin-bound Fmoc-Aaa-OH **78** by DDQ was observed by analytical HPLC for the standard proteinogenic amino acids with a protected reactive functional group in their side chain (Entries I-XIII and X-XIII). However, insignificant decomposition or oxidation of resin-bound Fmoc-Met-OH by DDQ was observed by analytical HPLC (Entry IX).

5.3. Synthesis of Dienophile Derivatised Amino Acid

The established synthesis of Fmoc-Lys(*exo*-BCN)-OH **75** was developed to enable the site-specific incorporation of H-Lys(*exo*-BCN)-OH into proteins expressed in *Escherichia coli* and mammalian cells.[103] The activation of *exo*-BCN-OH **60** with *N*,*N*`-disuccinimidyl carbonate (DSC) in the presence of triethylamine was reported to give *exo*-BCN-OSu **80**. Successive derivatisation of Fmoc-Lys-OH hydrochloride with *exo*-BCN-OSu **80** in the presence of *N*,*N*-diisopropylethylamine (DIPEA) to give Fmoc-Lys(*exo*-BCN)-OH **75** was reported at 85 % yield (over two steps). Upon following the literature procedure with *endo*-BCN-OH **60** and an additional purification step Fmoc-Lys(*endo*-BCN)-OH **75** was isolated in 38 % overall yield (Scheme 33).



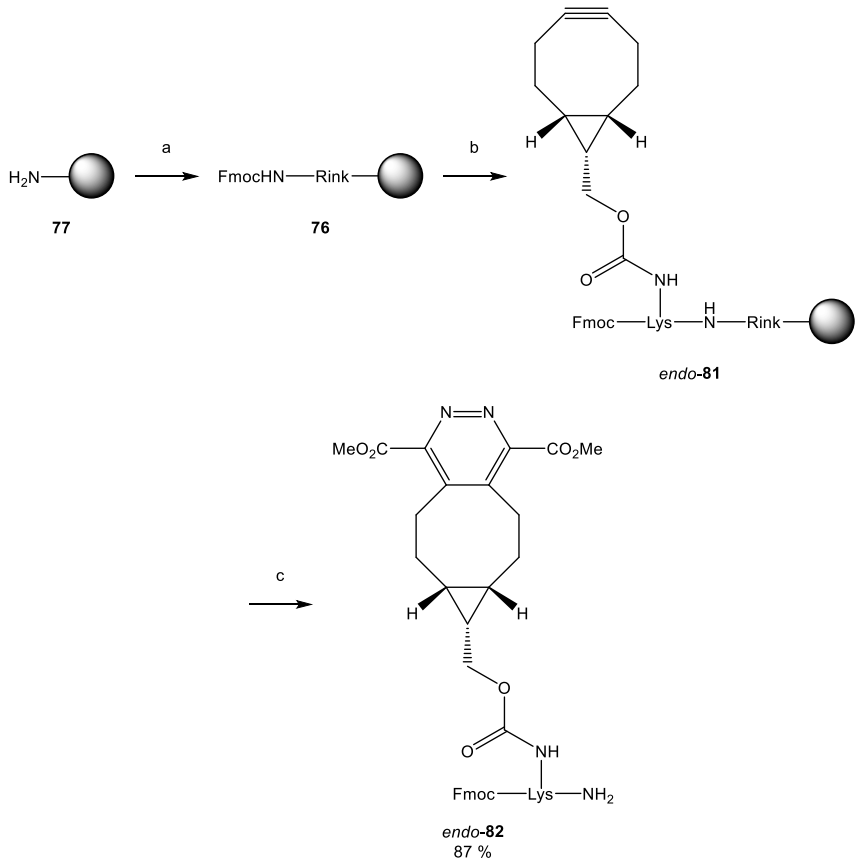
Scheme 33. Synthesis of Fmoc-Lys(*endo*-BCN)-OH **75**. Reagents and reaction conditions a) Et_3N (4 eq), DSC (1.5 eq), MeCN, rt, 4 h, N_2;[103] b) Fmoc-Lys-OH.HCl (1.5 eq), DIPEA (2 eq), DMF, rt, ~18 h, N_2.[103]

No synthesis was required for Fmoc-Asp(OAll)-OH and Fmoc-Glu(OAll)-OH as they were commercially available.

5.4. *In situ* Oxidation and Inverse Electron Demand Diels-Alder Reactions

The first optimised *in situ* oxidation and iedDA reaction of diester dihydrotetrazine **45** to be transferred to solid-phase was the *in situ* oxidation and iedDA reaction of diester dihydrotetrazine **45** with *endo*-BCN-OH **60** (Scheme 34). The resin functionalisation of amino polystyrene resin **77** with Rink amide linker in the presence of oxyma and DIC gave Rink amide polystyrene resin **76**. Successive Fmoc deprotection of Rink amide polystyrene resin **76** with piperidine and subsequent amino acid coupling of Fmoc-Lys(*endo*-BCN)-OH **75** in the presence of oxyma and DIC gave resin-bound Fmoc-Lys(*endo*-BCN)-OH **81**. The *in situ* oxidation and iedDA reaction of diester dihydrotetrazine **45** with resin-bound Fmoc-Lys(*endo*-BCN)-OH **81** in the presence of DDQ gave resin-bound *endo*-diester tricyclopyridazine derivatised Fmoc-Lys-OH. Successive linker cleavage of resin-bound *endo*-diester tricyclopyridazine derivatised Fmoc-Lys-OH with TFA:TIS:DCM to give *endo*-diester tricyclopyridazine derivatised Fmoc-Lys-NH_2 **82** was achieved in 84 % overall isolated yield. The *in situ* oxidation and iedDA reaction of diester dihydrotetrazine **45** with resin-bound Fmoc-Lys(*endo*-BCN)-OH **81** had been left for 4 h as the reaction could not be readily monitored. However, it was confirmed that the *in situ* oxidation and iedDA reaction of

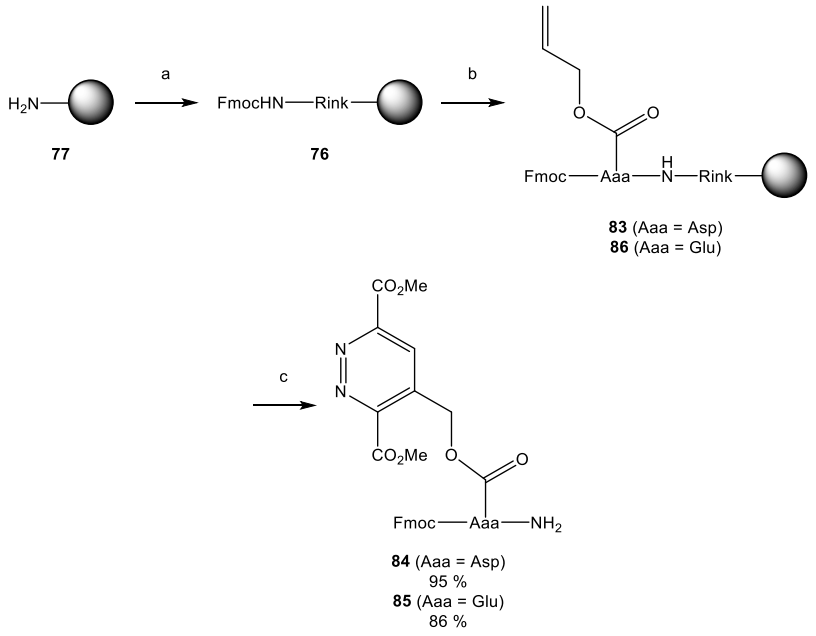
diester dihydrotetrazine **45** with resin-bound Fmoc-Lys(*endo*-BCN)-OH **81** had gone to completion as Fmoc-Lys(*endo*-BCN)-NH_2 was not observed in the crude material by analytical HPLC or LRMS. Also, no evidence of ester hydrolysis of *endo*-diester tricyclopyridazine derivatised Fmoc-Lys-NH_2 **82** by TFA was observed in the crude material by analytical HPLC or LRMS.



Scheme 34. *In situ* oxidation and iedDA reaction of diester dihydrotetrazine **45** with resin-bound Fmoc-Lys(*endo*-BCN)-OH **81** in the presence of DDQ. Reagents and reaction conditions a) Rink amide linker (3 eq), oxyma (3 eq), DIC (3 eq), DMF, rt, 45 min; b) i) piperidine in DMF, rt, 5 min; ii) Fmoc-Lys(*endo*-BCN)-OH **75** (3 eq), oxyma (3 eq), DIC (3 eq), DMF, rt, 45 min; c) i) diester dihydrotetrazine **45** (1.05 eq), DDQ (3 eq), DCM, rt, 4 h; ii) TFA:TIS:DCM (90:5:5), rt, 3 h.

The final optimised *in situ* oxidation and iedDA reaction of diester dihydrotetrazine **45** to be transferred to solid-phase was the *in situ* oxidation and iedDA reaction of diester dihydrotetrazine **45** with allyl ester **63** (Scheme 35). The resin functionalisation of amino polystyrene resin **77** with Rink amide linker in the presence of oxyma and

DIC gave Rink amide polystyrene resin **76**. Successive Fmoc deprotection of Rink amide polystyrene resin **76** with piperidine and subsequent amino acid coupling of Fmoc-Asp(OAll)-OH in the presence of oxyma and DIC gave resin-bound Fmoc-Asp(OAll)-OH **83**. The *in situ* oxidation and iedDA reaction of diester dihydrotetrazine **45** with resin-bound Fmoc-Asp(OAll)-OH **83** in the presence of DDQ gave resin-bound diester pyridazine derivatised Fmoc-Asp-OH. Successive linker cleavage of resin-bound diester pyridazine derivatised Fmoc-Asp-OH with TFA:TIS:DCM to give diester pyridazine derivatised Fmoc-Asp-NH_2 **84** was achieved in 95 % overall isolated yield. The *in situ* oxidation and iedDA reaction of diester dihydrotetrazine **45** with resin-bound Fmoc-Asp(OAll)-OH **83** had been left for 4 h as the reaction could not be readily monitored. However, it was confirmed that the *in situ* oxidation and iedDA reaction of diester dihydrotetrazine **45** with resin-bound Fmoc-Asp(OAll)-OH **83** had gone to completion as Fmoc-Asp(OAll)-NH_2 was not observed in the crude material by analytical HPLC or LRMS. Also, no evidence of ester hydrolysis of diester pyridazine derivatised Fmoc-Asp-NH_2 **84** by TFA was observed in the crude material by analytical HPLC or LRMS. The analogous solid-phase synthesis; *in situ* oxidation and iedDA reaction; and linker cleavage with Fmoc-Glu(OAll)-OH to give diester pyridazine derivatised Fmoc-Glu-NH_2 **85** was achieved in 86 % overall isolated yield. As expected, no observable differences were encountered between the *in situ* oxidation and iedDA reactions of diester dihydrotetrazine **45** with resin-bound Fmoc-Asp(OAll)-OH **83** and Fmoc-Glu(OAll)-OH **86**.

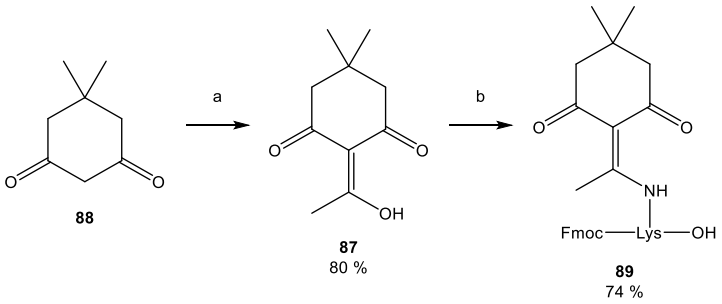


Scheme 35. *In situ* oxidation and iedDA reactions of diester dihydrotetrazine **45** with resin-bound Fmoc-Asp(OAll)-OH **83** and Fmoc-Glu(OAll)-OH **86** in the presence of DDQ. Reagents and reaction conditions a) Rink amide linker (3 eq), oxyma (3 eq), DIC (3 eq), DMF, rt, 45 min; b) i) piperidine in DMF, rt, 5 min; ii) Fmoc-Asp(OAll)-OH or Fmoc-Glu(OAll)-OH (3 eq), oxyma (3 eq), DIC (3 eq), DMF, rt, 45 min; c) i) diester dihydrotetrazine **45** (1.05 eq), DDQ (3 eq), DCM, rt, 4 h; ii) TFA:TIS:DCM (90:5:5), rt, 3 h.

5.5. Unsuccessful Synthesis of Resin-Bound Dihydro-1,2,4,5-tetrazine Derivatised Amino Acid

The 1-(4,4-dimethyl-2,6-dioxocyclohexylidene)ethyl (Dde) protecting group was developed as a novel amine protecting group which is orthogonal to both the Fmoc and *tert*-butyloxycarbonyl (Boc) protecting groups.[126] However, Fmoc deprotection occurs during the reported Dde deprotection procedure with hydrazine. Therefore, an alternative Dde deprotection procedure was reported within the literature which uses $NH_2OH.HCl$:imidazole to ensure full orthogonality between the Dde and Fmoc protecting groups.[127] Fmoc-Lys(Dde)-OH was selected for the late stage incorporation of the dihydro-1,2,4,5-tetrazine moiety onto the peptide backbone as it was commercially available; and due to the orthogonality of the Dde protecting group to both the Fmoc and TFA-labile protecting groups.

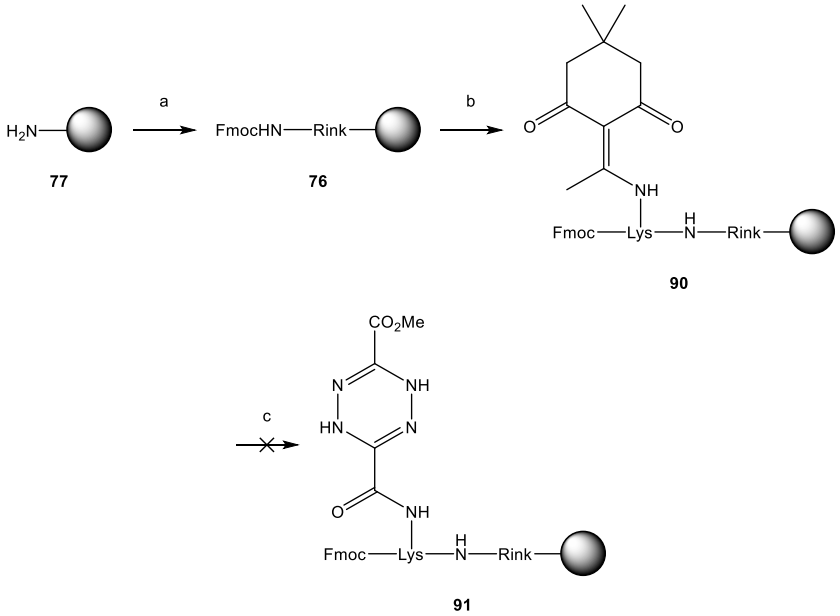
The established synthesis of Dde-OH **87** was developed to enable novel variants of the Dde protecting group to be synthesised with increased stability towards piperidine which also overcome *N*-to-*N*` intramolecular migration.[128,129] The acylation of cycloalkane diketone **88** with acetic acid in the presence of 4-(dimethylamino)pyridine (DMAP) and *N*-(3-dimethylaminopropyl)-*N*`-ethylcarbodiimide hydrochloride (EDC) to give Dde-OH **87** was reported at 84 % yield. Upon following the literature procedure Dde-OH **87** was isolated in 80 % yield (Scheme 36). The condensation of Dde-OH **87** with Fmoc-Lys-OH hydrochloride in the presence of DIPEA to give Fmoc-Lys(Dde)-OH **89** was achieved in 59 % overall isolated yield.



Scheme 36. Synthesis of Fmoc-Lys(Dde)-OH **89**. Reagents and reaction conditions a) DMAP (1.05 eq), EDC (1.05 eq), AcOH (1.05 eq), DMF, rt, 1 d;[129] b) Fmoc-Lys-OH.HCl (0.67 eq), DIPEA (1 eq), MeOH, rt, ~18 h.

The first attempted syntheses of resin-bound diester dihydrotetrazine derivatised amino acids were based on the bisamidation of diester dihydrotetrazine **45** with primary or secondary amines which had been reported within the literature.[20] The resin functionalisation of amino polystyrene resin **77** with Rink amide linker in the presence of oxyma and DIC gave Rink amide polystyrene resin **76** (Scheme 37). Successive Fmoc deprotection of Rink amide polystyrene resin **76** with piperidine and subsequent amino acid coupling of Fmoc-Lys(Dde)-OH **89** in the presence of oxyma and DIC gave resin-bound Fmoc-Lys(Dde)-OH **90**. The Dde deprotection of resin-bound Fmoc-Lys(Dde)-OH **90** with NH_2OH.HCl:imidazole and subsequent amidation of diester dihydrotetrazine **45** with resin-bound Fmoc-Lys-OH was proposed to give resin-bound diester dihydrotetrazine derivatised Fmoc-Lys-OH **91**. However, complete amidation was not observed based on the qualitative ninhydrin test even after

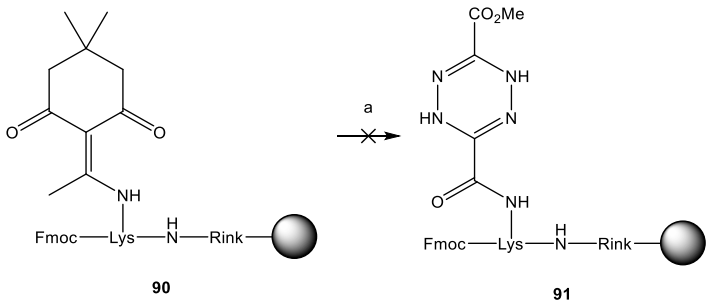
2 d at reflux in DMF with 6 eq of diester dihydrotetrazine **45**. Again, complete amidation was not observed based on the qualitative ninhydrin test for the analogous solid-phase synthesis and amidation with Fmoc-Gly-OH (Appendix 2). Therefore, no further attempts were made to synthesise resin-bound diester dihydrotetrazine derivatised amino acids based on the bisamidation of diester dihydrotetrazine **45** with primary or secondary amines.



Scheme 37. Unsuccessful synthesis of resin-bound diester dihydrotetrazine derivatised Fmoc-Lys-OH **91**. Reagents and reaction conditions a) Rink amide linker (3 eq), oxyma (3 eq), DIC (3 eq), DMF, rt, 45 min; b) i) piperidine in DMF, rt, 5 min; ii) Fmoc-Lys(Dde)-OH **89** (3 eq), oxyma (3 eq), DIC (3 eq), DMF, rt, 45 min; c) i) $NH_2OH.HCl$:imidazole (1:0.75) in DCM:NMP, rt, 3 h; ii) diester dihydrotetrazine **43** (6 eq), DMF, reflux, 2 d.

The last attempted syntheses of resin-bound diester dihydrotetrazine derivatised amino acids were based on the coupling of amino acids. The Dde deprotection of resin-bound Fmoc-Lys(Dde)-OH **90** with $NH_2OH.HCl$:imidazole and subsequent coupling of dicarboxylic acid dihydrotetrazine **48** with resin-bound Fmoc-Lys-OH in the presence of oxyma and DIC; and esterification of the second activated carboxylic acid with methanol in the presence of DIPEA was proposed to give resin-bound diester dihydrotetrazine derivatised Fmoc-Lys-OH **91** (Scheme 38). However, complete coupling of dicarboxylic acid dihydrotetrazine **48** was not observed based on the

qualitative ninhydrin test even after four coupling attempts. Again, complete coupling was not observed based on the qualitative ninhydrin test for the analogous solid-phase synthesis, coupling and esterification with Fmoc-Gly-OH (Appendix 3). Therefore, no further attempts were made to synthesise resin-bound diester dihydrotetrazine derivatised amino acids based on the coupling of amino acids.

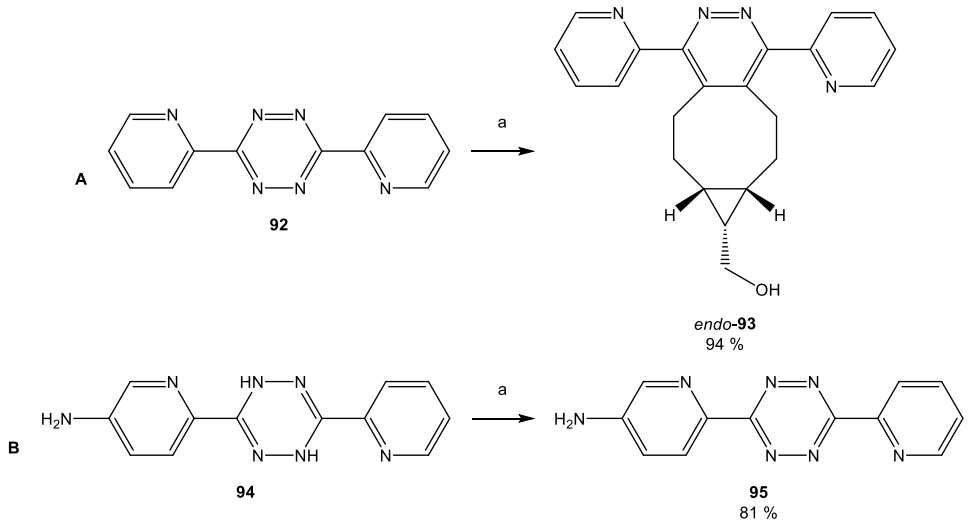


Scheme 38. Unsuccessful synthesis of resin-bound diester dihydrotetrazine derivatised Fmoc-Lys-OH **91**. Reagents and reaction conditions a) i) $NH_2OH.HCl$:imidazole (1:0.75) in DCM:NMP, rt, 3 h; ii) dicarboxylic acid dihydrotetrazine **48** (3 eq), oxyma (6 eq), DIC (6 eq), DMF, rt, 45 min; iii) DCM:DIPEA:MeOH (80:5:15), rt, 45 min.

5.6. Alternative Dihydro-1,2,4,5-tetrazine

Qualitative screening studies of the reactivity of a library of 1,2,4,5-tetrazines with a library of dienophiles were conducted to identify candidate 1,2,4,5-tetrazine-dienophile pairs for the solid-phase thermal activation peptide macrocyclisation strategy (Appendix 4-8). However, dipyridyl tetrazine **92** fulfilled the requirements as an alternative dihydro-1,2,4,5-tetrazine for the solid-phase oxidation activation peptide macrocyclisation strategy. The iedDA reaction of dipyridyl tetrazine **92** with *endo*-BCN-OH **60** had gone to completion within 15 min as indicated by the loss of purple colour from dipyridyl tetrazine **92**. To corroborate the qualitative result *endo*-dipyridyl tricyclopyridazine alcohol **93** was isolated in 94 % yield (Scheme 39A). The oxidation of amino dipyridyl dihydrotetrazine **94** with DDQ to give amino dipyridyl tetrazine **95** has been reported at 81 % yield (Scheme 39B).[75] Also, the amine group of amino dipyridyl dihydrotetrazine **94** provides a functional handle which can be used for the late stage incorporation of amino dipyridyl dihydrotetrazine **94** onto the peptide

backbone.[81] Therefore, amino dipyridyl dihydrotetrazine **94** was selected as an alternative dihydro-1,2,4,5-tetrazine for future development of the solid-phase oxidation activation peptide macrocyclisation strategy.



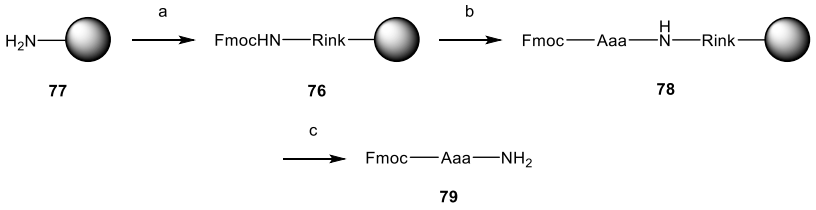
Scheme 39A. iedDA reaction of dipyridyl tetrazine **92** with *endo*-BCN-OH **60**. Reagents and reaction conditions a) *endo*-BCN-OH **60** (1.05 eq), DCM, rt, 2 h. **Scheme 39B.** Reported synthesis of amino dipyridyl tetrazine **95**. Reagents and reaction conditions a) DDQ (2 eq), toluene, reflux, 12 h, N_2.[75]

5.7. Conclusions

The aims of this chapter were to transfer the optimised *in situ* oxidation and iedDA reactions of diester dihydrotetrazine **45** to solid-phase and to verify the *in situ* oxidation and iedDA reactions of resin-bound diester dihydrotetrazine derivatised amino acids in the presence of DDQ so that peptide macrocyclisations can be conducted on solid-phase.

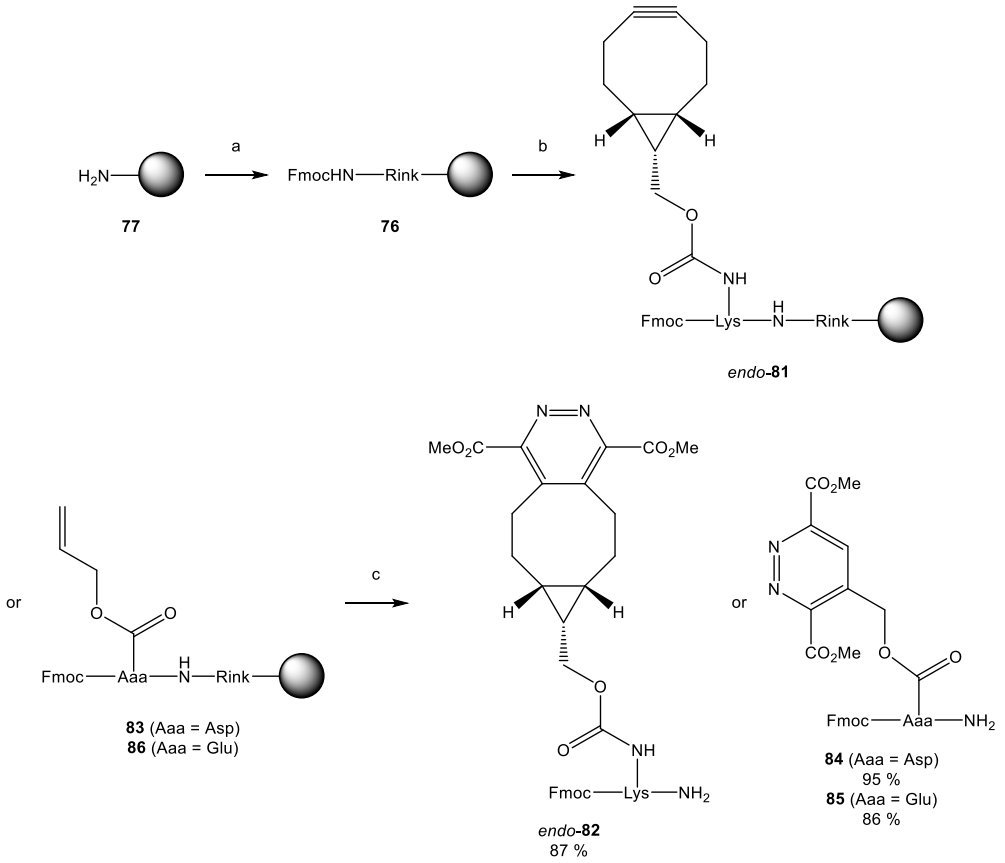
Commercially available N_α-Fmoc protected standard proteinogenic amino acids were selected for the investigation into the stability of resin-bound amino acids towards DDQ as these are commonly used amino acids in SPPS and therefore to be used for peptide macrocyclisation. No decomposition of resin-bound Fmoc-Aaa-OH **78** by DDQ was observed by analytical HPLC for the standard proteinogenic amino acids with a protected reactive functional group in their side chain (Scheme 40). However,

insignificant decomposition or oxidation of resin-bound Fmoc-Met-OH by DDQ was observed by analytical HPLC.



Scheme 40. Investigation into the stability of resin-bound amino acids towards DDQ. Reagents and reaction conditions a) Rink amide linker (3 eq), oxyma (3 eq), DIC (3 eq), DMF, rt, 45 min; b) i) piperidine in DMF, rt, 5 min; ii) Fmoc-Aaa-OH (3eq), oxyma (3 eq), DIC (3 eq), DMF, rt, 45 min; c) i) DDQ (3 eq), DCM, rt, 8 h; ii) TFA:TIS:DCM (90:5:5), rt, 1 h.

The *in situ* oxidation and iedDA reactions of diester dihydrotetrazine **45** with resin-bound Fmoc-Lys(*endo*-BCN)-OH **81**, Fmoc-Asp(OAll)-OH **83** and Fmoc-Glu(OAll)-OH **86** in the presence of DDQ to give *endo*-diester tricyclopyridazine derivatised Fmoc-Lys-NH_2 **82**, diester pyridazine derivatised Fmoc-Asp-NH_2 **84** and diester pyridazine derivatised Fmoc-Glu-NH_2 **85** were achieved in 87, 95 and 86 % isolated yield respectively (Scheme 41). Therefore, the optimised *in situ* oxidation and iedDA reactions of diester dihydrotetrazine **45** were successfully transferred to solid-phase.



Scheme 41. *In situ* oxidation and iedDA reactions of diester dihydrotetrazine **45** with resin-bound Fmoc-Lys(*endo*-BCN)-OH **81**, Fmoc-Asp(OAll)-OH **83** and Fmoc-Glu(OAll)-OH **86** in the presence of DDQ. Reagents and reaction conditions a) Rink amide linker (3 eq), oxyma (3 eq), DIC (3 eq), DMF, rt, 45 min; b) i) piperidine in DMF, rt, 5 min; ii) Fmoc-Lys(*endo*-BCN)-OH **75**, Fmoc-Asp(OAll)-OH or Fmoc-Glu(OAll)-OH (3 eq), oxyma (3 eq), DIC (3 eq), DMF, rt, 45 min; c) i) diester dihydrotetrazine **45** (1.05 eq), DDQ (3 eq), DCM, rt, 4 h; ii) TFA:TIS:DCM (90:5:5), rt, 3 h.

The late stage incorporation of diester dihydrotetrazine **45** onto the peptide backbone was not successfully verified due to unsuccessful attempts to synthesise resin-bound diester dihydrotetrazine derivatised amino acids.

The *in situ* oxidation and iedDA reactions of resin-bound diester dihydrotetrazine derivatised amino acids in the presence of DDQ were not successfully verified due to unsuccessful attempts to synthesise resin-bound diester dihydrotetrazine derivatised amino acids. The oxidation of amino dipyridyl dihydrotetrazine **94** with DDQ to give amino dipyridyl tetrazine **95** has been reported at 81 % yield.[75] Also, the amine group of amino dipyridyl dihydrotetrazine **94** provides a functional handle which can be used

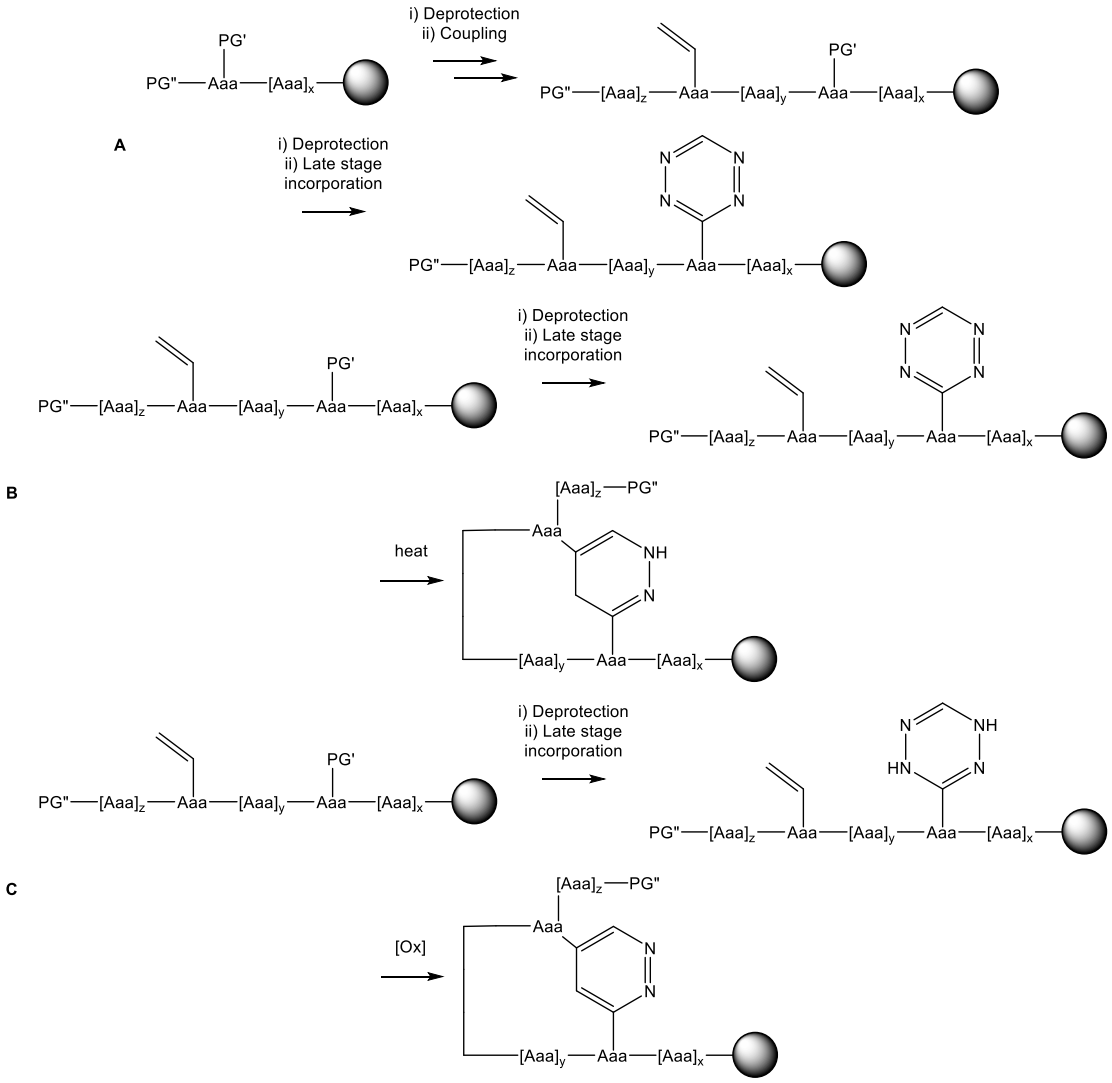
for the late stage incorporation of amino dipyridyl dihydrotetrazine **94** onto the peptide backbone.[81] Therefore, amino dipyridyl dihydrotetrazine **94** was selected as an alternative dihydro-1,2,4,5-tetrazine for future development of the solid-phase oxidation activation peptide macrocyclisation strategy.

Chapter 6. Conclusions and Future Work

6.1. Conclusions

The aim of this thesis was to develop a novel solid-phase peptide macrocyclisation strategy based on the iedDA reaction of 1,2,4,5-tetrazines. A solid-phase peptide macrocyclisation strategy was selected due to their amenability to automated and combinatorial synthesis; and efficiency and simplicity over solution-phase strategies.[87-89] Also, the pseudodilution effect can be exploited to favour intramolecular over intermolecular reactions in a solid-phase peptide macrocyclisation strategy.[90-92] The iedDA reaction of 1,2,4,5-tetrazines was selected due to its efficiency, orthogonality, tunability and versatility as shown by its successful application in numerous fields of research.[69,75,76,79-86]

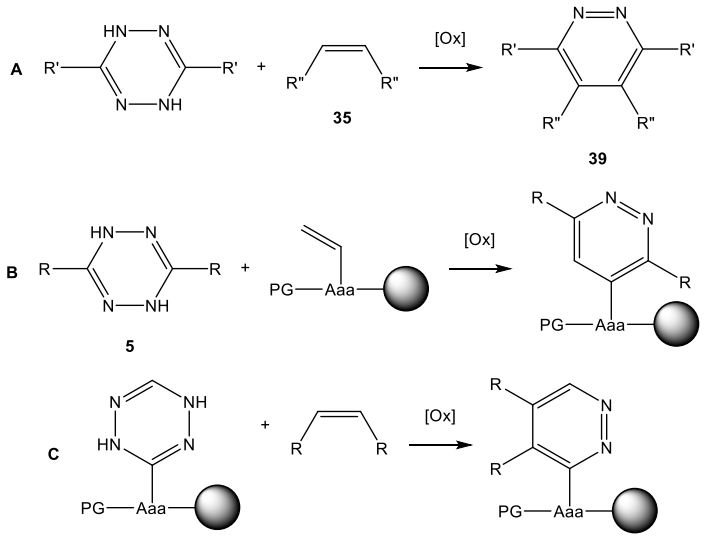
It was decided to use an orthogonal deprotection strategy to enable late stage incorporation of the 1,2,4,5-tetrazine moiety onto the peptide backbone due to the then unknown stability of 1,2,4,5-tetrazines towards the deprotection, coupling and cleavage cycles of SPPS (Scheme 42A).[93] A strategy was required to prevent any undesired iedDA reaction during the late stage incorporation of the 1,2,4,5-tetrazine moiety onto the peptide backbone in the presence of a dienophile. The solid-phase thermal activation peptide macrocyclisation strategy was proposed to prevent any undesired iedDA reaction during the late stage incorporation of the 1,2,4,5-tetrazine moiety onto the peptide backbone in the presence of a dienophile (Scheme 42B). However, the fine balance of reaction rates required for selective late stage incorporation of the 1,2,4,5-tetrazine moiety onto the peptide backbone in the presence of a dienophile and complete control over peptide macrocyclisation would be difficult to realise. Therefore, the solid-phase oxidation activation peptide macrocyclisation strategy was selected over the solid-phase thermal activation peptide macrocyclisation strategy (Scheme 42C). The solid-phase oxidation activation peptide macrocyclisation strategy enables selective late stage incorporation of the dihydro-1,2,4,5-tetrazine moiety onto the peptide backbone in the presence of a dienophile and complete control over peptide macrocyclisation.



Scheme 42A. Orthogonal deprotection strategy to enable late stage incorporation of the 1,2,4,5-tetrazine moiety onto the peptide backbone. **Scheme 42B.** Solid-phase thermal activation peptide macrocyclisation strategy. **Scheme 42C.** Solid-phase oxidation activation peptide macrocyclisation strategy.

To successfully develop a novel solid-phase peptide macrocyclisation strategy based on the *in situ* oxidation and iedDA reaction of a dihydro-1,2,4,5-tetrazine several prerequisites needed to be achieved. Firstly, a proof of concept *in situ* oxidation and iedDA reaction of a dihydro-1,2,4,5-tetrazine needed to be conducted to determine the viability of the solid-phase oxidation activation peptide macrocyclisation strategy (Scheme 43A). Secondly, optimisation of the proof of concept *in situ* oxidation and iedDA reaction of a dihydro-1,2,4,5-tetrazine would be required before transferring

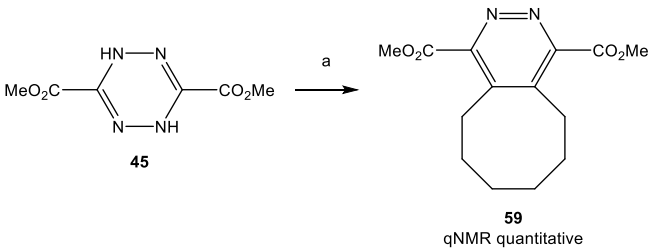
the optimised *in situ* oxidation and iedDA reaction of a dihydro-1,2,4,5-tetrazine to solid-phase and verifying the *in situ* oxidation and iedDA reactions of resin-bound dihydro-1,2,4,5-tetrazine derivatised amino acids (Schemes 43B and 43C respectively). Finally, peptide macrocyclisations needed to be conducted to determine the scope and limitations of the solid-phase oxidation activation peptide macrocyclisation strategy.



Scheme 43A. *In situ* oxidation and iedDA reaction of a dihydro-1,2,4,5-tetrazine. **Scheme 43B.** *In situ* oxidation and iedDA reaction of a dihydro-1,2,4,5-tetrazine transferred to solid-phase. **Scheme 43C.** *In situ* oxidation and iedDA reaction of a resin-bound dihydro-1,2,4,5-tetrazine derivatised amino acid.

Diester dihydrotetrazine **45** was selected for the proof of concept *in situ* oxidation and iedDA reaction of a dihydro-1,2,4,5-tetrazine as its synthesis had been previously reported within the literature;[22] it can be readily oxidised to the reactive diester tetrazine **46** with isopentyl nitrite which is amenable to SPPS;[80] and the amidation of diester dihydrotetrazine **45** with primary or secondary amines can be used for the late stage incorporation of diester dihydrotetrazine **45** onto the peptide backbone. (*E*)-Cycloalkyne **60** was selected as the model dienophile for the proof of concept *in situ* oxidation and iedDA reaction of a dihydro-1,2,4,5-tetrazine due to its improved isolated yield over the analogous iedDA reactions of diester tetrazine **46** with (*Z*)-bicycloalkene **51** and (*E*)-cycloalkene **49**. The *in situ* oxidation and iedDA reaction of

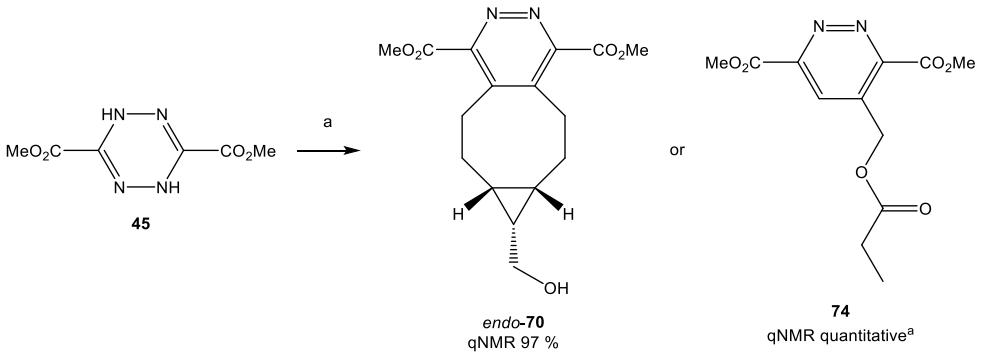
diester dihydrotetrazine **45** with cycloalkyne **50** in the presence of isopentyl nitrite to give diester bicyclopyridazine **59** was achieved in quantitative proton NMR yield (Scheme 44). Therefore, a successful proof of concept *in situ* oxidation and iedDA reaction of a dihydro-1,2,4,5-tetrazine was achieved. However, the *in situ* oxidation and iedDA reaction of diester dihydrotetrazine **45** with cycloalkyne **50** in the presence of isopentyl nitrite also highlighted the poor stability of a strained dienophile towards an oxidant.



Scheme 44. *In situ* oxidation and iedDA reaction of diester dihydrotetrazine **45** with cycloalkyne **50** in the presence of isopentyl nitrite. Reagents and reaction conditions a) cycloalkyne **50** (1.05 eq), isopentyl nitrite (25 eq), DCM, rt, 2 h.

Two strategies were proposed to overcome the poor stability of a strained dienophile towards an oxidant and therefore optimise the proof of concept *in situ* oxidation and iedDA reaction of a dihydro-1,2,4,5-tetrazine. Firstly, an alternative oxidant which does not facilitate decomposition of a strained dienophile can overcome the poor stability of a strained dienophile towards an oxidant. DDQ, BTI and PTAD were selected as alternative oxidants to optimise the proof of concept *in situ* oxidation and iedDA reaction of a dihydro-1,2,4,5-tetrazine due to their corroborated quantitative qNMR yields in the oxidation of diester dihydrotetrazine **45**. Secondly, an unstrained dienophile which is stable to decomposition by an oxidant can overcome the poor stability of a strained dienophile towards an oxidant. *endo*-BCN-OH **60** and allyl ester **63** were selected as dienophiles to optimise the proof of concept *in situ* oxidation and iedDA reaction of a dihydro-1,2,4,5-tetrazine due to their corroborated quantitative and near quantitative qNMR yield respectively in the iedDA reactions of diester tetrazine **46**. The *in situ* oxidation and iedDA reactions of diester dihydrotetrazine **45** with *endo*-BCN-OH **60** and allyl ester **63** in the presence of DDQ to give *endo*-diester

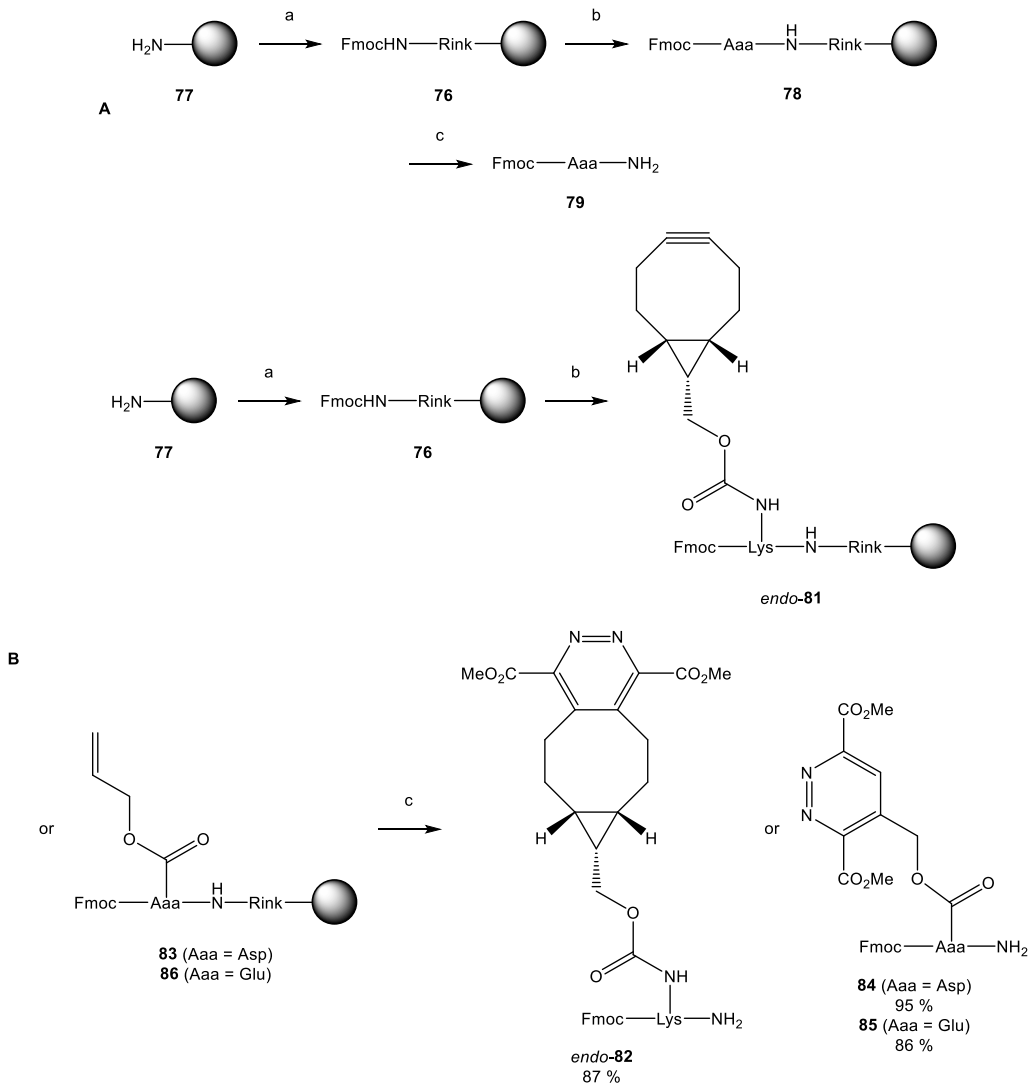
tricyclopyridazine alcohol **70** and diester pyridazine ester **74** were achieved in 97 % and quantitative qNMR yield respectively (Scheme 45). Therefore, the proof of concept *in situ* oxidation and iedDA reaction of a dihydro-1,2,4,5-tetrazine was successfully optimised in the presence of an alternative oxidant and with an unstrained dienophile in the presence of the alternative oxidant.



Scheme 45. *In situ* oxidation and iedDA reactions of diester dihydrotetrazine **45** with *endo*-BCN-OH **60** and allyl ester **63** in the presence of DDQ. Reagents and reaction conditions a) durene (0.5 eq), *endo*-BCN-OH **60** or allyl ester **63** (1.05 eq), DDQ (3 eq), DCM-d_2, 25 °C, 2 h. [a] Diester pyridazine ester **74** was isolated in 97 % yield.

The stability of resin-bound amino acids towards DDQ were determined before transferring the optimised *in situ* oxidation and iedDA reactions of diester dihydrotetrazine **45** to solid-phase and verifying the *in situ* oxidation and iedDA reactions of resin-bound diester dihydrotetrazine derivatised amino acids in the presence of DDQ. No decomposition of resin-bound Fmoc-Aaa-OH **78** by DDQ was observed by analytical HPLC for the standard proteinogenic amino acids with a protected reactive functional group in their side chain (Scheme 46A). However, insignificant decomposition or oxidation of resin-bound Fmoc-Met-OH by DDQ was observed by analytical HPLC. The *in situ* oxidation and iedDA reactions of diester dihydrotetrazine **45** with resin-bound Fmoc-Lys(*endo*-BCN)-OH **81**, Fmoc-Asp(OAll)-OH **83** and Fmoc-Glu(OAll)-OH **86** in the presence of DDQ to give *endo*-diester tricyclopyridazine derivatised Fmoc-Lys-NH_2 **82**, diester pyridazine derivatised Fmoc-Asp-NH_2 **84** and diester pyridazine derivatised Fmoc-Glu-NH_2 **85** were achieved in 87, 95 and 86 % isolated yield respectively (Scheme 46B). Therefore,

the optimised *in situ* oxidation and iedDA reactions of diester dihydrotetrazine **45** were successfully transferred to solid-phase. However, the *in situ* oxidation and iedDA reactions of resin-bound diester dihydrotetrazine derivatised amino acids in the presence of DDQ were not successfully verified due to unsuccessful attempts to synthesise resin-bound diester dihydrotetrazine derivatised amino acids.

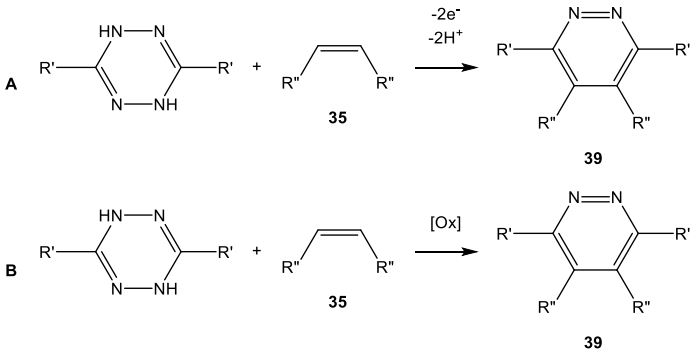


Scheme 46A. Investigation into the stability of resin-bound amino acids towards DDQ. Reagents and reaction conditions a) Rink amide linker (3 eq), oxyma (3 eq), DIC (3 eq), DMF, rt, 45 min; b) i) piperidine in DMF, rt, 5 min; ii) Fmoc-Aaa-OH (3eq), oxyma (3 eq), DIC (3 eq), DMF, rt, 45 min; c) i) DDQ (3 eq), DCM, rt, 8 h; ii) TFA:TIS:DCM (90:5:5), rt, 1 h. **Scheme 46B.** *In situ* oxidation and iedDA reactions of diester dihydrotetrazine **45** with resin-bound Fmoc-Lys(*endo*-BCN)-OH **81**, Fmoc-Asp(OAll)-OH **83** and Fmoc-Glu(OAll)-OH **86** in the presence of DDQ. Reagents and reaction

conditions a) Rink amide linker (3 eq), oxyma (3 eq), DIC (3 eq), DMF, rt, 45 min; b) i) piperidine in DMF, rt, 5 min; ii) Fmoc-Lys(*endo*-BCN)-OH **75**, Fmoc-Asp(OAll)-OH or Fmoc-Glu(OAll)-OH (3 eq), oxyma (3 eq), DIC (3 eq), DMF, rt, 45 min; c) i) diester dihydrotetrazine **45** (1.05 eq), DDQ (3 eq), DCM, rt, 4 h; ii) TFA:TIS:DCM (90:5:5), rt, 3 h.

In conclusion, a successful proof of concept *in situ* oxidation and iedDA reaction of a dihydro-1,2,4,5-tetrazine was achieved which determined the viability of the solid-phase oxidation activation peptide macrocyclisation strategy. The proof of concept *in situ* oxidation and iedDA reaction of a dihydro-1,2,4,5-tetrazine was successfully optimised before successfully transferring the optimised *in situ* oxidation and iedDA reactions of a dihydro-1,2,4,5-tetrazine to solid-phase. However, the *in situ* oxidation and iedDA reactions of resin-bound diester dihydrotetrazine derivatised amino acids in the presence of DDQ were not successfully verified due to unsuccessful attempts to synthesise resin-bound diester dihydrotetrazine derivatised amino acids. Therefore, no peptide macrocyclisations were conducted to determine the scope and limitations of the solid-phase oxidation activation peptide macrocyclisation strategy.

An electrochemical strategy to selectively trigger the iedDA reaction of a 1,2,4,5-tetrazine has been previously reported within the literature (Scheme 47A).[130] However, a non-electrochemical strategy to selectively trigger the iedDA reaction of a 1,2,4,5-tetrazine has not been reported within the literature. The *in situ* oxidation and iedDA reaction of a dihydro-1,2,4,5-tetrazine methodology reported in this thesis currently provides the only non-electrochemical strategy to selectively trigger the iedDA reaction of a 1,2,4,5-tetrazine (Scheme 47B). The non-electrochemical strategy to selectively trigger the iedDA reaction of a 1,2,4,5-tetrazine could find applications in material, polymer and supramolecular chemistry. However, careful selection of the dihydro-1,2,4,5-tetrazine, dienophile and oxidant would be required for successful application of the non-electrochemical strategy to selectively trigger the iedDA reaction of a 1,2,4,5-tetrazine in material, polymer or supramolecular chemistry.

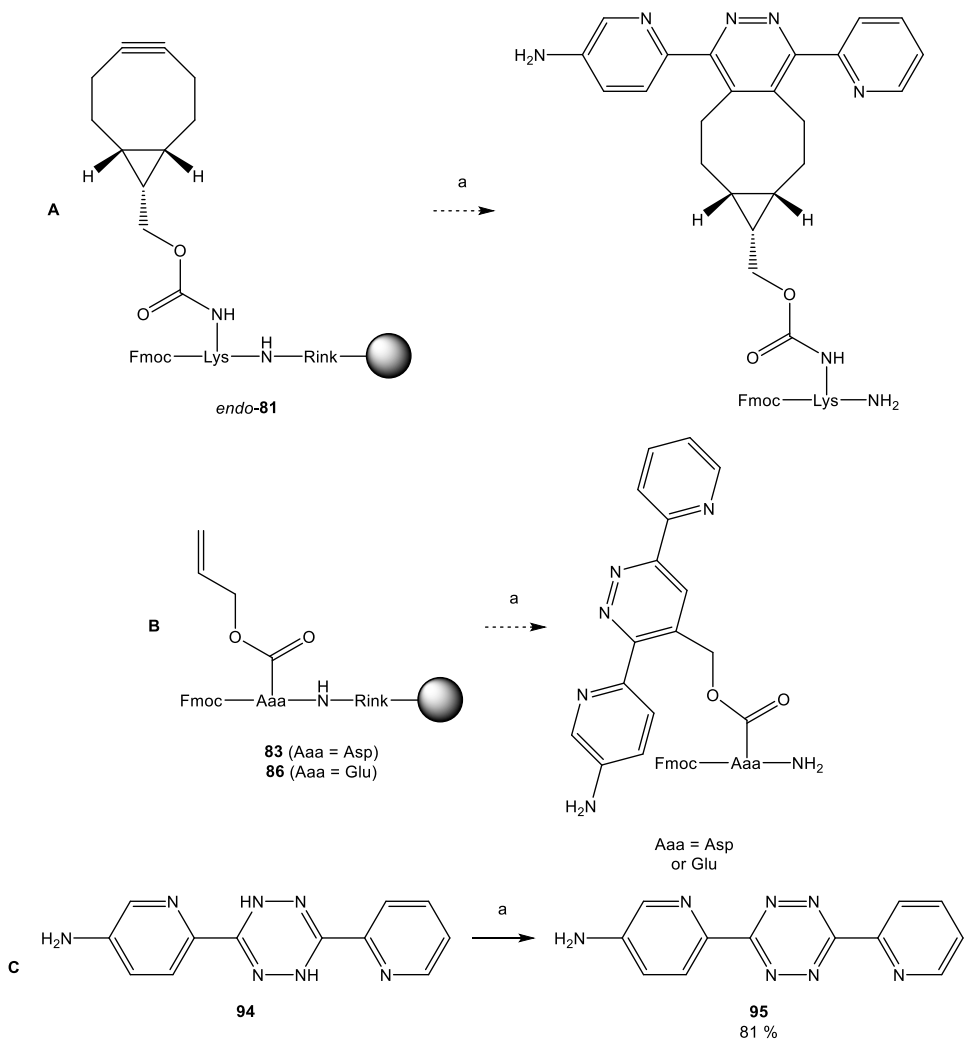


Scheme 47A. Electrochemical strategy to selectively trigger the iedDA reaction of a 1,2,4,5-tetrazine.[130] **Scheme 47B.** Non-electrochemical strategy to selectively trigger the iedDA reaction of a 1,2,4,5-tetrazine.

6.2. Future Work

To successfully develop a novel solid-phase peptide macrocyclisation strategy based on the *in situ* oxidation and iedDA reaction of a dihydro-1,2,4,5-tetrazine several prerequisites still need to be achieved.

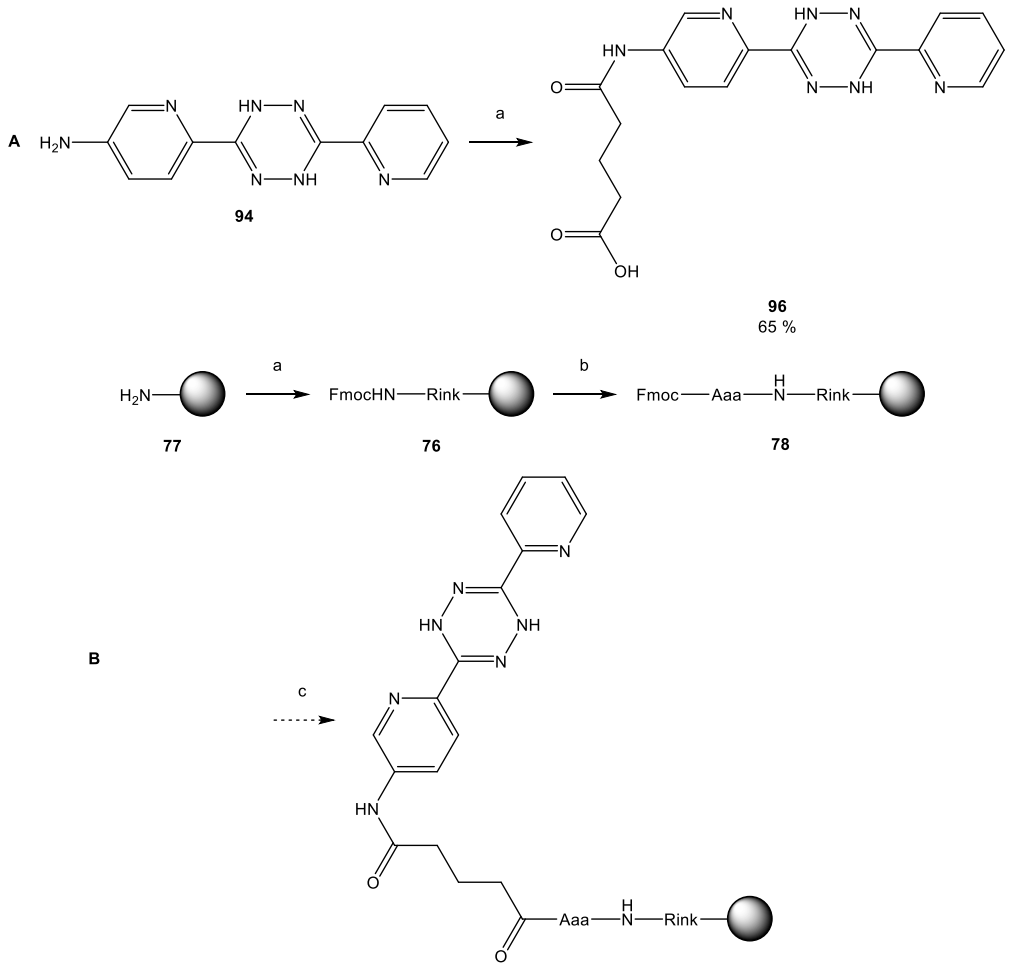
Firstly, the solid-phase *in situ* oxidation and iedDA reactions of diester dihydrotetrazine **45** with resin-bound Fmoc-Lys(*endo*-BCN)-OH **81**; and Fmoc-Asp(OAll)-OH **83** and Fmoc-Glu(OAll)-OH **86** in the presence of DDQ need to be repeated with amino dipyridyl dihydrotetrazine **94** to verify the solid-phase *in situ* oxidation and iedDA reactions of amino dipyridyl dihydrotetrazine **94** (Schemes 48A and 48B respectively). The oxidation of amino dipyridyl dihydrotetrazine **94** with DDQ to give amino dipyridyl tetrazine **95** has been reported at 81 % yield (Scheme 48C).[75]



Scheme 48A. Proposed *in situ* oxidation and iedDA reaction of amino dipyridyl dihydrotetrazine **94** with resin-bound Fmoc-Lys(*endo*-BCN)-OH **81** in the presence of DDQ. Reagents and reaction conditions a) i) amino dipyridyl dihydrotetrazine **94** (1.05 eq), DDQ (3 eq), DCM, rt, 4 h; ii) TFA:TIS:DCM (90:5:5), rt, 3 h. **Scheme 48B.** Proposed *in situ* oxidation and iedDA reaction of amino dipyridyl dihydrotetrazine **94** with Fmoc-Asp(OAll)-OH **83** and Fmoc-Glu(OAll)-OH **86** in the presence of DDQ. Reagents and reaction conditions a) i) amino dipyridyl dihydrotetrazine **94** (1.05 eq), DDQ (3 eq), DCM, rt, 4 h; ii) TFA:TIS:DCM (90:5:5), rt, 3 h. **Scheme 48C.** Reported synthesis of amino dipyridyl tetrazine **95**. Reagents and reaction conditions a) DDQ (2 eq), toluene, reflux, 12 h, N_2.[75]

Secondly, resin-bound amino dipyridyl dihydrotetrazine derivatised amino acids need to be synthesised to verify the late stage incorporation of amino dipyridyl dihydrotetrazine **94** onto the peptide backbone. The amine group of amino dipyridyl

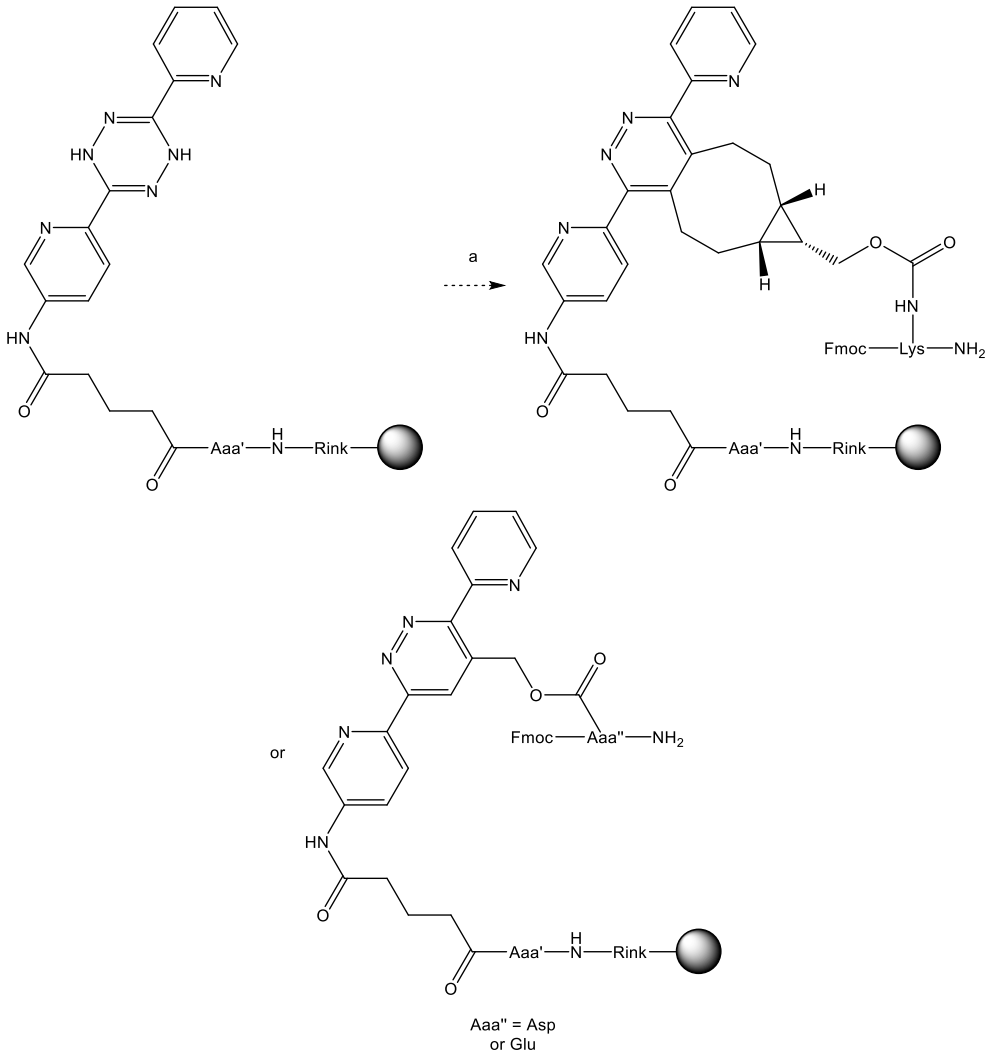
dihydrotetrazine **94** provides a functional handle which can be used for the late stage incorporation of amino dipyridyl dihydrotetrazine **94** onto the peptide backbone.[81] The amidation of glutaric anhydride with amino dipyridyl dihydrotetrazine **94** has been reported to give carboxylic acid dipyridyl dihydrotetrazine **96** at 65 % yield (Scheme 49A). The amidation of carboxylic acid dipyridyl dihydrotetrazine **96** with primary and secondary amines in the presence oxyma and DIC was proposed to give resin-bound amino dipyridyl dihydrotetrazine derivatised amino acids (Scheme 49B). Therefore, the amidation of carboxylic acid dipyridyl dihydrotetrazine **96** with primary and secondary amines in the presence oxyma and DIC was selected for the synthesis of resin-bound amino dipyridyl dihydrotetrazine derivatised amino acids.



Scheme 49A. Reported synthesis of carboxylic acid dipyridyl dihydrotetrazine **96**. Reagents and reaction conditions a) glutaric anhydride (5 eq), THF, reflux, 20 h. **Scheme 49B.** Proposed synthesis of resin-bound amino dipyridyl dihydrotetrazine derivatised amino acids. Reagents and reaction

conditions a) Rink amide linker (3 eq), oxyma (3 eq), DIC (3 eq), DMF, rt, 45 min; b) i) piperidine in DMF, rt, 5 min; ii) Fmoc-Aaa-OH (3eq), oxyma (3 eq), DIC (3 eq), DMF, rt, 45 min; c) i) piperidine in DMF, rt, 5 min; ii) carboxylic acid dipyridyl dihydrotetrazine **96** (3 eq), oxyma (6 eq), DIC (6 eq), DMF, rt, 45 min.

Thirdly, the *in situ* oxidation and iedDA reactions of resin-bound amino dipyridyl dihydrotetrazine derivatised amino acids with Fmoc-Lys(*endo*-BCN)-OH **75**, Fmoc-Asp(OAll)-OH and Fmoc-Glu(OAll)-OH in the presence of DDQ need to be conducted to verify the *in situ* oxidation and iedDA reactions of resin-bound amino dipyridyl dihydrotetrazine derivatised amino acids (Scheme 50).



Scheme 50. Proposed *in situ* oxidation and iedDA reactions of resin-bound amino dipyridyl dihydrotetrazine derivatised amino acid with Fmoc-Lys(*endo*-BCN)-OH **75**, Fmoc-Asp(OAll)-OH

and Fmoc-Glu(OAll)-OH in the presence of DDQ. Reagents and reaction conditions a) i) Fmoc-Lys(*endo*-BCN)-OH **75**, Fmoc-Asp(OAll)-OH and Fmoc-Glu(OAll)-OH (1.05 eq), DDQ (3 eq), DCM, rt, 4 h; ii) TFA:TIS:DCM (90:5:5), rt, 3 h.

Finally, peptide macrocyclisations need to be conducted to determine the scope and limitations of the solid-phase oxidation activation peptide macrocyclisation strategy.

Potential research projects which have arisen from the work presented in this thesis include research projects to develop the non-electrochemical strategy to selectively trigger the iedDA reaction of a 1,2,4,5-tetrazine for use in material, polymer and supramolecular chemistry.

Chapter 7. Experimental

7.1. Materials and Methods

Materials

All commercially available solvents and reagents were used as obtained from the supplier unless otherwise stated. Distilled water was obtained from an ELGA Option 3 Water Purification System. Anhydrous acetonitrile, DCM, diethyl ether, methanol and tetrahydrofuran (THF) were obtained using a Glass Contour Bench Top Solvent Purification System.

Solution-Phase Synthesis

Non-anhydrous solution-phase synthesis was conducted using standard laboratory glassware and equipment; and standard non-anhydrous techniques. Anhydrous solution-phase synthesis was conducted using oven dried laboratory glassware, standard laboratory equipment and standard anhydrous techniques under a positive pressure of nitrogen.

Solid-Phase Synthesis

Solid-phase synthesis was conducted using column reservoirs fitted with polyethylene frits and Teflon stopcocks; GL Biochem Aminomethyl Polystyrene Resin (100-200 Mesh, 0.3-0.8 $mmolg^{-1}$, 1 % DVB); standard laboratory equipment; and standard solid-phase techniques. Amino acid couplings were tested for completion using a modified version of the qualitative ninhydrin test.[131] Solution A (3 drops) and solution B (1 drop) were added to a few beads of resin at rt. The resin was maintained at 110 °C for 3 min. Solution A was prepared by adding potassium cyanide (1.30 mg) in water (2 mL), ethanol (10 mL) and phenol (40 mL) to freshly distilled pyridine (100 mL) at rt. Solution B was prepared by adding ninhydrin (2.50 g) to ethanol (50 mL) at rt.

Chromatography

Thin-layer chromatography (TLC) was conducted using Merck TLC Silica Gel 60 F_{254} Plates and visualised by eye; potassium permanganate and heating; or short wave ultraviolet light (254 nm). Potassium permanganate stain was prepared by adding potassium permanganate (1.50 g), potassium carbonate (10.0 g) and sodium hydroxide solution (1.25 mL, 3 M aq) to water (200 mL) at rt.

Flash column chromatography was conducted using Merck Silica Gel 60 according to the procedure of Still *et al.*[132] The crude products were dry loaded onto the column using Fisher Chemical General Purpose Grade Kieselguhr Washed with Acid then eluted using an isocratic solvent system or a step graduated solvent system with increasing solvent polarity.

Automated flash column chromatography was conducted using a Teledyne Isco CombiFlash R_f Flash Chromatography System with detection at 254 and 280 nm; and Teledyne RediSep R_f Normal-Phase Silica Columns. The crude products were dry loaded onto the column using Sigma Aldrich Filter Agent then eluted using an isocratic solvent system or a graduated solvent system with increasing solvent polarity.

Melting Point

Melting points were recorded in open capillary tubes using a Gallenkamp Melting Point Apparatus and are uncorrected.

Infrared Spectroscopy

Infrared (IR) spectra were recorded using a Bruker Tensor 27 FT-IR Spectrometer fitted with a Specac Golden Gate Single Reflection Diamond ATR Attachment or a Perkin Elmer Spectrum 65 FT-IR Spectrometer fitted with a PIKE MIRacle single reflection ZnSe Crystal Plate. Each spectrum was recorded with a scan range of 4500-500 cm^{-1} using 16 background scans of a clean crystal plate and 32 scans of sample at

4 cm^{-1} resolution. Only frequencies corresponding to significant functional groups are reported.

Nuclear Magnetic Resonance Spectroscopy

Proton NMR spectra were recorded at 25 degree Celsius (°C) using an automated Bruker Ascend 500 MHz Spectrometer fitted with a Bruker DCH-ATMA CryoProbe, an automated Bruker Ascend 500 MHz Spectrometer fitted with a Bruker Prodigy BBO CryoProbe, an automated Bruker Avance I 400 MHz Spectrometer fitted with a Bruker BBI Probe, an automated Bruker Avance I 400 MHz Spectrometer fitted with a Bruker QNP Probe, an automated Bruker Avance III 400 MHz Spectrometer fitted with a Bruker BBFO-ATMA Probe, an automated Bruker Avance III 400 MHz Spectrometer fitted with a Bruker PABBO-BB Probe or an automated Bruker Avance III 500 MHz Spectrometer fitted with a Bruker DCH-ATMA CryoProbe in deuterated chloroform, deuterated dichloromethane (DCM-d_2) or deuterated dimethyl sulfoxide (DMSO-d_6) using residual non-deuterated solvent as the internal reference. The chemical shifts are reported in parts per million (ppm).

Carbon NMR spectra were recorded at 25 °C using an automated Bruker Ascend 500 MHz Spectrometer fitted with a Bruker DCH-ATMA CryoProbe, an automated Bruker Avance I 400 MHz Spectrometer fitted with a Bruker QNP Probe, an automated Bruker Avance III 400 MHz Spectrometer fitted with a Bruker PABBO-BB Probe or an automated Bruker Avance III 500 MHz Spectrometer fitted with a Bruker DCH-ATMA CryoProbe in deuterated chloroform or DMSO-d_6 using deuterated solvent as the internal reference. The chemical shifts are reported in ppm.

qNMR yields were calculated from separated peaks in proton NMR spectra recorded without any modifications to the open access proton NMR experimental parameters and the relative quantitation method (Equation 1).[133]

$$n_{anal} = \frac{I_{anal}}{I_{int}} \times \frac{N_{int}}{N_{anal}} \times n_{int}$$

n_{anal} = number of moles of analyte

I_{anal} = integral of analyte signal

I_{int} = integral of qNMR internal standard signal

N_{int} = number of qNMR internal standard protons

N_{anal} = number of analyte protons

n_{int} = number of moles of qNMR internal standard

Equation 1. Relative quantitation method.

Mass Spectroscopy

Low-resolution mass spectra were recorded using an Agilent 1100 HPLC Modular System coupled to an Agilent Technologies 6130 LC/MS Quadrupole Mass Spectrometer, a Bruker MicrOTOF Mass Spectrometer or a Hewlett-Packard 1100 LC/MSD Quadrupole Mass Spectrometer under electrospray ionisation (ESI) conditions; a Thermo Finnigan LCQ Mass Spectrometer under ESI or electron ionisation (EI) conditions; or a Thermo Finnigan MAT 900 XLP Mass Spectrometer under chemical ionisation (CI), EI, ESI or fast atom bombardment (FAB) conditions.

High-resolution mass spectra were recorded using an Agilent 1100 HPLC Modular System coupled to a Bruker MicrOTOF Mass Spectrometer under ESI conditions or a Thermo Finnigan MAT 900 XLP Mass Spectrometer under CI, EI or ESI conditions.

High-Performance Liquid Chromatography

Analytical HPLC was conducted using an Agilent Technologies 1100 Series HPLC Modular System coupled to a Polymer Laboratories PL-ELS 1000 Evaporative Light Scattering Detector and an Agilent Technologies 1100 Series Diode-Array Detector with detection at 254, 350, 500, 650 and 750 nm.

Column 1: Agilent Poroshell 120 SB-C18, 2.7 μm, 4.6 × 50 mm.

Column 2: Supelco Discovery HS C18 HPLC Column, 5 μm, 4.6 × 50 mm.

Column 3: Phenomenex Kinetex XB-C18, 5 μm, 4.6 × 50 mm.

Method A: Gradient 5.0 to 95 % (10 min), isocratic (4 min), gradient 95 to 5.0 % (5 s) then isocratic (55 s) at a flow rate of 1 millilitre (mL) per minute using HPLC grade acetonitrile with 0.10 % formic acid in HPLC grade water with 0.10 % formic acid.

Method B: Gradient 5.0 to 95 % (10 min), isocratic (4 min), gradient 95 to 5.0 % (5 s) then isocratic (55 s) at a flow rate of 1 $mLmin^{-1}$ using HPLC grade methanol with 0.10 % formic acid in HPLC grade water with 0.10 % formic acid.

7.2. General Procedures

General Procedure 1: Resin Functionalisation

Resin (1 eq) was swollen in DCM for 5 min then washed with DCM (× 3). Oxyma (3 eq) was added to a stirring solution of linker (3 eq) at rt in DMF. The reaction mixture was stirred for 5 min before adding DIC (3 eq). The reaction mixture was stirred for 2 min before adding to the resin. The resin was agitated for 45 min then filtered before washing with DMF (× 3), DCM (× 3), methanol (× 3) and diethyl ether (× 3). The functionalised resin was dried under reduced pressure prior to storage.

General Procedure 2: Fmoc Deprotection and Amino Acid Coupling

Functionalised resin (1 eq) was swollen in DCM for 5 min then washed with DCM (× 3). Piperidine solution (20 % DMF) was added to the functionalised resin at rt. The functionalised resin was agitated for 5 min then filtered before washing with DMF (× 3) and DCM (× 3). The Fmoc deprotection step was repeated twice. Oxyma (3 eq) was added to a stirring solution of Fmoc-Aaa-OH (3 eq) at rt in DMF. The reaction mixture was stirred for 5 min before adding DIC (3 eq). The reaction mixture was stirred for 2 min before adding to the functionalised resin. The functionalised resin was agitated for

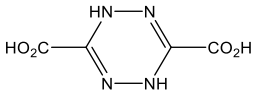
45 min then filtered before washing with DMF (× 3), DCM (× 3), methanol (× 3) and diethyl ether (× 3). The amino acid loaded functionalised resin was dried under reduced pressure prior to storage.

General Procedure 3: Linker Cleavage

Amino acid loaded functionalised resin (1 eq) was swollen in DCM for 5 min then washed with DCM (× 3). TFA:TIS:DCM (90:5:5) was added to the resin at rt. The amino acid loaded functionalised resin was agitated for 1-3 h then filtered before washing with TFA (× 3). The combined organic filtrates were concentrated under reduced pressure and the residue suspended in cold diethyl ether. The suspension was centrifuged then decanted before drying under reduced pressure to give crude product.

7.3. Chapter 3 Experimental

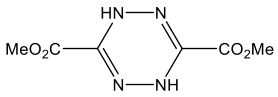
1,4-Dihydro-1,2,4,5-tetrazine-3,6-dicarboxylate 48[22]



Ethyl diazoacetate (4.61 mL, 43.8 mmol, 1 eq) was added dropwise to a stirring solution of sodium hydroxide (8.04 g, 0.201 mol, 4.6 eq) in water (12 mL) at rt. The reaction mixture was heated to 70 °C and stirred for 1.5 h before cooling to rt. Ethanol (50 mL) was added to the reaction mixture. The reaction mixture was stirred for 30 min before the insoluble solid was removed by filtration. The filtrand was washed with ethanol (3 × 20 mL) and diethyl ether (3 × 20 mL) then dried under reduced pressure. Hydrochloric acid (9.17 mL, 57 % aq) was added dropwise to a stirring solution of filtrand (4.52 g, 20.9 mmol, 1 eq) in water (10 mL) at -10 °C. The insoluble solid was immediately removed by filtration and the filtrand resuspended in water (12 mL) at 0 °C. The suspension was stirred for 30 min before the insoluble solid was removed by filtration. The filtrand was washed with cold water (3 × 10 mL) and cold diethyl ether (3 × 10 mL) then dried under reduced pressure to give dicarboxylic acid

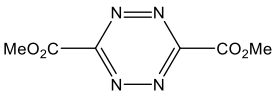
dihydrotetrazine **48** as a yellow solid (1.33 g, 36 %): **R_f** (DCM:MeOH, 7:3) 0.32; **mp** 145-146 °C, literature 144-148 °C;[22] **IR** (neat, cm^{-1}) 3531 (O-H), 3318 (N-H), 1696 (C=O), 1186 (C-O); **^{1}H NMR** δ (500 MHz, DMSO-d_6) 8.96 (2H, s, RN*H*N); **^{13}C NMR** δ (125 MHz, DMSO-d_6) 161.3 (2C, R*C*O_2H), 140.8 (2C, N*C*NHCO$_2$H); ***m/z*** (ESI-, DMSO) 170.8 ([M-H]$^-$, 24 %), 126.8 (28), 61.9 (100); **HRMS** (ESI-, DMSO) not observed; **HPLC** (column 1, method B, ELSD, H_2O) t_R 1.03 min.

3,6-Dimethyl 1,4-Dihydro-1,2,4,5-tetrazine-3,6-dicarboxylate 45[22]



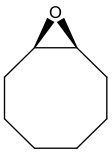
Freshly distilled thionyl chloride (4.24 mL, 59.8 mmol, 2.6 eq) was added dropwise to stirring anhydrous methanol (25 mL) at -30 °C under nitrogen. Dicarboxylic acid dihydrotetrazine **48** (3.95 g, 23.0 mmol, 1 eq) in anhydrous methanol (32 mL) was added dropwise to the thionyl chloride solution. The reaction mixture was heated to 40 °C and stirred for 2 h before cooling to -30 °C. The insoluble solid was removed by filtration and the filtrand washed with cold methanol (3 × 10 mL) and cold diethyl ether (3 × 10 mL) before drying under reduced pressure to give crude product. The crude product was purified by flash column chromatography eluting with DCM to give diester dihydrotetrazine **45** as an orange solid (3.55 g, 77 %): **R_f** (DCM:MeOH, 19:1) 0.68; **mp** 170-171 °C, literature 170-171 °C;[22] **IR** (neat, cm^{-1}) 3359 (N-H), 1720 (C=O), 1198 (C-O); **^{1}H NMR** δ (500 MHz, $CDCl_3$) 7.47 (2H, s, RN*H*N), 3.92 (6H, s, CO_2CH_3); **^{13}C NMR** δ (125 MHz, $CDCl_3$) 159.2 (2C, R*C*O_2R), 138.2 (2C, N*C*NHCO$_2$R), 53.9 (2C, CO_2CH_3); ***m/z*** (ESI+, MeOH) 201.2 ([M+H]$^+$, 52 %), 222.8 ([M+Na]$^+$, 7 %), 423.2 ([2M+Na]$^+$, 50 %), 127.2 (30), 115.2 (100), 105.2 (34); **HPLC** (column 1, method B, ELSD, MeOH) t_R 0.78 min. The spectroscopic data was in agreement with the literature.[22]

3,6-Dimethyl 1,2,4,5-Tetrazine-3,6-dicarboxylate 46[80]



Isopentyl nitrite (0.505 mL, 3.75 mmol, 3 eq) was added dropwise to a stirring solution of diester dihydrotetrazine **45** (0.250 g, 1.25 mmol, 1 eq) in DCM (10 mL) at rt. The reaction mixture was stirred for 2 h before the solvent was removed under reduced pressure to give crude product. The crude product was purified by recrystallisation in DCM:hexane (10:1) to give diester tetrazine **46** as a red solid (0.119 g, 48 %): **R_f** decomposed on TLC plate; **mp** 174-175 °C, literature 173-175 °C;[22] **IR** (neat, cm^{-1}) 1745 (C=O), 1206 (C-O); **^{1}H NMR** δ (500 MHz, $CDCl_3$) 4.23 (6H, s, CO_2CH_3); **^{13}C NMR** δ (125 MHz, $CDCl_3$) 160.5 (2C, N*C*NCO$_2$R), 159.3 (2C, Ar*C*O$_2$R), 54.8 (2C, CO_2CH_3); ***m/z*** (EI, DCM) 198.0 ($[M]^{+\cdot}$, 54 %), 54.0 (100); **HPLC** (column 1, method A, ELSD, MeCN) t_R 0.76 min. The spectroscopic data was in agreement with the literature.[22]

9-Oxabicyclo[6.1.0]nonane 53[108]



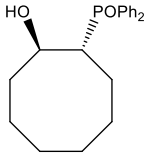
cis-Cyclooctene (11.8 mL, 90.7 mmol, 1 eq) was added to a stirring solution of *m*CPBA (22.8 g, 90.7 mmol, 1 eq) in DCM (200 mL) at rt. The reaction mixture was stirred for 5 h. The reaction mixture was washed with sodium carbonate solution (3 × 200 mL, sat aq) and water (3 × 200 mL) then dried over anhydrous magnesium sulfate before the solvent was removed under reduced pressure to give crude product. The crude product was purified by flash column chromatography eluting with diethyl ether:hexane (1:19) to give cycloalkane epoxide **53** as a colourless solid (7.50 g, 65 %): **R_f** (Et_2O:hexane, 1:19) 0.31; **mp** 53-54 °C, literature 56-57 °C;[134] **IR** (neat, cm^{-1}) no reportable peaks observed; **^{1}H NMR** δ (500 MHz, $CDCl_3$) 2.92-2.88 (2H, m,

RC*H*OR), 2.14-2.12 (2H, m, RCHOCH_2R), 1.63-1.40 (8H, m, RCH_2R), 1.31-1.23 (2H, m, RCHOCH_2R); **^{13}C NMR** δ (125 MHz, $CDCl_3$) 55.8 (2C, R*C*HOR), 26.7 (2C, R*C*H_2R), 26.4 (2C, RCHO*C*H_2R), 25.7 (2C R*C*H_2R); ***m/z*** (ESI+, DCM) 127.0 ($[M+H]^+$, 7 %), 149.0 ($[M+Na]^+$, 61 %), 133.0 (26), 119.0 (28), 65.0 (100); **HPLC** (column 1, method B, ELSD, MeOH) t_R not observed. The spectroscopic data was in agreement with the literature.[108]

0.674 M Lithiodiphenylphosphane Solution 97[108]

Chlorodiphenylphosphine (16.8 mL, 90.6 mmol, 1 eq) in anhydrous THF (39 mL) was added dropwise to lithium wire (1.38 g, 0.199 mol, 2.2 eq) in anhydrous THF (95 mL) at rt under nitrogen. The reaction mixture was stirred overnight to give phosphane solution **97** which was used without characterisation.

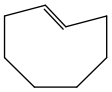
(±)-*trans*-2-(Diphenylphosphoroso)cyclooctan-1-ol 54[108]



Cycloalkane epoxide **53** (11.3 g, 89.5 mmol, 1 eq) in anhydrous THF (42 mL) was added to stirring phosphane solution **97** (146 mL, 98.5 mmol, 1.1 eq) at rt under nitrogen. The reaction mixture was stirred for 2 d before acetic acid (6.41 mL, 0.112 mol, 1.25 eq) and hydrogen peroxide solution (133 mL, 3 % aq) were added dropwise at 0 °C. The reaction mixture was warmed to rt and stirred for 1 h before extracting with DCM (3 × 300 mL). The combined organic extracts were washed with water (3 × 300 mL) then dried over anhydrous magnesium sulfate before the solvent was removed under reduced pressure to give crude product. The crude product was purified by flash column chromatography eluting with DCM to give cycloalkane epoxide **53** (1.92 g, 17 %). Further elution with DCM:methanol (49:1) gave (±)-*trans*-alcohol

phosphine oxide cycloalkane **54** as a colourless solid (21.1 g, 72 %): **R_f** (DCM:MeOH, 49:1) 0.19; **mp** 160-161 °C, literature 149-150 °C, benzene;[108] **IR** (neat, cm^{-1}) 3248 (O-H), 1115 (C-O); **^{1}H NMR** δ (500 MHz, $CDCl_3$) 7.80-7.71 (4H, m, Ar*H*), 7.60-7.55 (2H, m, Ar*H*), 7.52-7.48 (4H, m, Ar*H*), 4.34 (1H, br s, RO*H*), 4.13-4.09 (1H, m, RC*H*OHR), 2.81-2.74 (1H, m, RC*H*PR), 1.96-1.56 (8H, m, RCH_2CHOHR, RCHPCH_2R and RCH_2R), 1.49-1.33 (3H, m, RCH_2R), 1.23-1.19 (1H, m, RCHPCH_2R); **^{13}C NMR** δ (125 MHz, $CDCl_3$) 132.8 (2C, d, *J* 8.4 Hz, ArH), 132.3 (1C, d, *J* 2.6 Hz, ArH), 132.3 (1C, d, *J* 2.6 Hz, ArH), 132.1 (1C, d, *J* 96.5 Hz, ArP), 131.3 (2C, d, *J* 9.1 Hz, ArH), 129.4 (1C, d, *J* 93.5 Hz, ArP), 128.9 (2C, d, *J* 11.4 Hz, ArH), 128.5 (2C, d, *J* 11.1 Hz, ArH), 70.5 (1C, d, *J* 3.5 Hz, R*C*HOHR), 42.6 (1C, d, *J* 67.5 Hz, R*C*HPR), 30.8 (1C, d, *J* 12.4 Hz, R*C*H_2CHOHR), 29.0 (1C, d, *J* 10.6 Hz, R*C*H_2R), 27.0 (1C, R*C*H_2R), 25.0 (1C, R*C*H_2R), 24.6 (1C, RCHP*C*H_2R), 21.2 (1C, R*C*H_2R); ***m/z*** (ESI+, MeCN) 329.2 ($[M+H]^+$, 32 %), 351.2 ($[M+Na]^+$, 17 %), 657.4 ($[2M+H]^+$, 17 %), 679.4 ($[2M+Na]^+$, 100 %), 146.2 (76), 105.2 (58); **HPLC** (column 1, method A, ELSD, MeCN) t_R 7.61 min. The spectroscopic data was in agreement with the literature.[108]

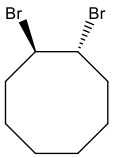
(*E*)-Cyclooctene 49[108]



(±)-*trans*-Alcohol phosphine oxide cycloalkane **54** (8.42 g, 25.6 mmol, 1 eq) in anhydrous DMF (70 mL) was added dropwise to a stirring solution of sodium hydride (0.737 g, 30.7 mmol, 1.2 eq) in anhydrous DMF (85 mL) at 0 °C under nitrogen. The reaction mixture was warmed to rt and stirred for 3 h before diluting with pentane (200 mL). The reaction mixture was washed with ammonium chloride solution (1 × 200 mL, sat aq) and water (3 × 200 mL) then dried over anhydrous magnesium sulfate before the solvent was removed under reduced pressure to give crude product. The crude product was purified by distillation under reduced pressure to give (*E*)-cycloalkene **49** as a colourless oil (1.11 g, 39 %): **$dr_{E:Z}$** 99:1; **R_f** (hexane) 0.71; **bp** 54-55 °C, 40 mbar, literature 44 °C, 30 mbar;[104] **IR** (neat, cm^{-1}) 1650 (C=C); **^{1}H NMR** δ

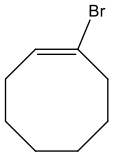
(500 MHz, $CDCl_3$) 5.51-5.46 (2H, m, RC*H*R), 2.38-2.35 (2H, m, RCHCH_2R), 2.00-1.91 (4H, m, RCHCH_2R and RCH_2R), 1.86-1.78 (2H, m, RCH_2R), 1.47-1.39 (2H, m, RCH_2R), 0.83-0.76 (2H, m, RCH_2R); **^{13}C NMR** δ (125 MHz, $CDCl_3$) 134.1 (2C, R*C*HR), 35.9 (2C, RCH*C*H_2R), 35.8 (2C, R*C*H_2R), 29.3 (2C, R*C*H_2R); ***m/z*** (EI, hexane) 110.1 ($[M]^{+\cdot}$, 39 %), 67.1 (100); **HPLC** (column 1, method B, ELSD, MeOH) t_R not observed. The spectroscopic data was in agreement with the literature.[108]

(±)-*trans*-1,2-Dibromocyclooctane 55[110]



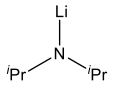
Bromine (3.94 mL, 76.8 mmol, 1 eq) was added dropwise to a stirring solution of *cis*-cyclooctene (10.0 mL, 76.8 mmol, 1 eq) in DCM (30 mL) at -40 °C. The reaction mixture was stirred at -40 °C for 30 min before warming to rt. The reaction mixture was washed with sodium thiosulfate solution (1 × 30 mL, sat aq), water (1 × 30 mL) and sodium chloride solution (1 × 30 mL, sat aq) then dried over anhydrous magnesium sulfate before the solvent was removed under reduced pressure to give crude product. The crude product was purified by flash column chromatography eluting with hexane to give (±)-*trans*-dibromocycloalkane **55** as a colourless oil (11.2 g, 54 %): **R_f** (hexane) 0.39; **IR** (neat, cm^{-1}) 696 (C-Br); **^{1}H NMR δ** (400 MHz, $CDCl_3$) 4.61-4.56 (2H, m, RC*H*BrR), 2.44-2.38 (2H, m, RCHBrCH_2R), 2.10-2.05 (2H, m, RCHBrCH_2R), 1.90-1.81 (2H, m, RCH_2R), 1.72-1.55 (4H, m, RCH_2R), 1.52-1.45 (2H, m, RCH_2R); **^{13}C NMR δ** (125 MHz, $CDCl_3$) 61.7 (2C, R*C*HBrR), 33.4 (2C, RCHBr*C*H_2R), 26.1 (2C, R*C*H_2R), 25.6 (2C, R*C*H_2R); ***m/z*** (CI, hexane) 269.9 ($[M+H]^+$, 76 %), 268.9 (74); **HPLC** (column 1, method B, ELSD, MeOH) t_R not observed. The spectroscopic data was in agreement with the literature.[135]

(1*E*)-1-Bromocyclooct-1-ene 56[110]



1 M Potassium *tert*-butoxide solution in anhydrous THF (5.55 mL, 5.55 mmol, 1.5 eq) was added dropwise to a stirring solution of (±)-*trans*-dibromocycloalkane **55** (1.00 g, 3.70 mmol, 1 eq) in anhydrous diethyl ether (1.5 mL) at 0 °C under nitrogen. The reaction mixture was warmed to rt and stirred for 1 h before diluting with water (20 mL). The organic layer was separated from the aqueous (aq) layer and the aq layer extracted with diethyl ether (3 × 20 mL). The organic layer and combined organic extracts were dried over anhydrous magnesium sulfate before the solvent was removed under reduced pressure to give crude product. The crude product was purified by flash column chromatography eluting with hexane to give (*E*)-bromocycloalkene **56** as a colourless oil (0.453 g, 65 %): **R$_f$** (hexane) 0.63; **IR** (neat, cm^{-1}) 1642 (C=C); **^{1}H NMR δ** (400 MHz, $CDCl_3$) 6.03 (1H, t, *J* 8.5 Hz, RC*H*R), 2.63-2.60 (2H, m, RCBrC*H*$_2$R), 2.10-2.07 (2H, m, RC*H*$_2$CHR), 1.65-1.61 (2H, m, RC*H*$_2$R), 1.57-1.50 (6H, m, RC*H*$_2$R); **^{13}C NMR δ** (125 MHz, $CDCl_3$) 131.8 (1C, R*C*HR), 125.0 (1C, R*C*BrR), 35.3 (1C, RCBr*C*H$_2$R), 30.0 (1C, R*C*H$_2$R), 28.8 (1C, R*C*H$_2$CHR), 27.6 (1C, R*C*H$_2$R), 26.5 (1C, R*C*H$_2$R), 25.6 (1C, R*C*H$_2$R); ***m/z*** (EI, hexane) 190.0 ([M]$^{+\cdot}$, 28 %), 188.0 (29), 109.0 (78), 67.0 (100); **HPLC** (column 1, method B, ELSD, MeOH) t_R not observed. The spectroscopic data was in agreement with the literature.[136]

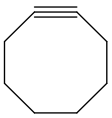
0.834 M Lithium Diisopropylamide Solution 98[110]



1.6 M *n*-Butyllithium solution in anhydrous hexanes (14.5 mL, 23.2 mmol, 0.5 eq) was added dropwise to a stirring solution of freshly distilled diisopropylamine (3.38 mL,

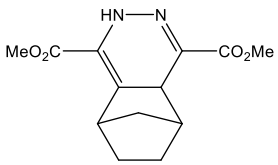
24.1 mmol, 0.52 eq) in anhydrous THF (11 mL) at -25 °C under nitrogen. LDA solution **98** was used immediately without characterisation.

Cyclooctyne 50[110]



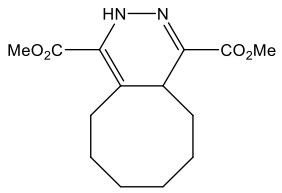
(*E*)-Bromocycloalkene **56** (8.78 g, 48.2 mmol, 1 eq) was added to LDA solution **98** (28.9 mL, 24.1 mmol, 0.5 eq) at -25 °C under nitrogen. The reaction mixture was warmed to rt and stirred for 2.5 h before neutralising with hydrochloric acid (35 mL, 1 M aq). The organic layer was separated from the aq layer and the aq layer extracted with hexane (3 × 50 mL). The organic layer and combined organic extracts were washed with water (3 × 50 mL) then dried over anhydrous magnesium sulfate before the solvent was removed under reduced pressure to give crude product. The crude product was purified by distillation under reduced pressure to give cycloalkyne **50** as a colourless oil (0.394 g, 16 %): **R_f** (hexane) 0.81; **bp** 59-61 °C, 40 mbar, literature 51-52 °C, 29 mbar;[110] **IR** (neat, cm^{-1}) no reportable peaks observed; **^{1}H NMR δ** (500 MHz, $CDCl_3$) 2.17-2.14 (4H, m, RCC*H*$_2$R), 1.90-1.82 (4H, m, RC*H*$_2$R), 1.64-1.60 (4H, m, RC*H*$_2$R); **^{13}C NMR δ** (125 MHz, $CDCl_3$) 94.9 (2C, R*C*R), 34.8 (2C, RC*C*H$_2$R), 30.0 (2C, R*C*H$_2$R), 21.2 (2C, R*C*H$_2$R); ***m/z*** (EI, hexane) 108.1 ([M]$^{+\cdot}$, 16 %), 79.1 (100); **HPLC** (column 1, method B, ELSD, MeOH) t_R not observed. The spectroscopic data was in agreement with the literature.[137]

3,6-Dimethyl 4,5-Diazatricyclo[6.2.1.0^{2,7}]undeca-2,5-diene-3,6-dicarboxylate 57



Bicyclo[2.2.1]hept-2-ene (37.5 mg, 0.398 mmol, 1.05 eq) was added slowly to a stirring solution of diester tetrazine **46** (75.0 mg, 0.379 mmol, 1 eq) in DCM (3.79 mL) at rt. The reaction mixture was stirred for 2 h before the solvent was removed under reduced pressure to give crude product. The crude product was purified by flash column chromatography eluting with ethyl acetate:hexane (1:9) to give diester tricyclodihydropyridazine **57** as a colourless solid (79.0 mg, 79 %): $\mathbf{R_f}$ (EtOAc:hexane, 1:3) 0.25; **mp** 122-123 °C, literature 122-123 °C, MeOH;[113] **IR** (neat, cm^{-1}) 3327 (N-H), 1695 (C=O), 1138 (C-O); **^{1}H NMR δ** (500 MHz, $CDCl_3$) 8.52 (1H, br s, RN*H*N), 3.86 (3H, s, CO_2CH_3), 3.83 (3H, s, CO_2CH_3), 3.61 (1H, d, *J* 3.9 Hz, R_3CH), 3.51 (1H, d, *J* 4.0 Hz, R_3CH), 2.09 (1H, d, *J* 1.9 Hz, R_3CH), 1.90-1.76 (2H, m, $RCH_{2eq}R$), 1.58-1.50 (2H, m, RCH_2R and $RCH_{2ax}R$), 1.38-1.33 (1H, m, $RCH_{2ax}R$), 1.19-1.16 (1H, m, RCH_2R); **^{13}C NMR δ** (125 MHz, $CDCl_3$) 164.3 (1C, RCO_2R), 162.1 (1C, RCO_2R), 132.4 (1C, $NCRCO_2R$), 128.7 (1C, $RCNHCO_2R$), 121.1 (1C, R_3C), 52.4 (1C, CO_2CH_3), 52.4 (1C, CO_2CH_3), 43.5 (1C, R_3CH), 42.3 (1C, RCH_2R), 41.4 (1C, R_3CH), 37.6 (1C, R_3CH), 29.0 (1C, RCH_2R), 26.4 (1C, RCH_2R); ***m/z*** (ESI+, MeCN) 265.2 ($[M+H]^+$, 17 %), 287.1 ($[M+Na]^+$, 13 %), 529.3 ($[2M+H]^+$, 13 %), 551.2 ($[2M+Na]^+$, 100 %); **HPLC** (column 1, method A, ELSD, MeCN) t_R 6.72 min. The spectroscopic data was in agreement with the literature.[113]

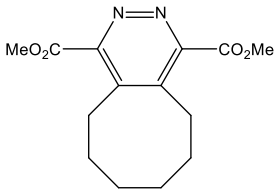
1,4-Dimethyl 2*H*,4a*H*,5*H*,6*H*,7*H*,8*H*,9*H*,10*H*-Cycloocta[d]pyridazine-1,4-dicarboxylate 58



(*E*)-Cycloalkene **49** (61.8 mg, 0.561 mmol, 1.05 eq) was added dropwise to a stirring solution of diester tetrazine **46** (0.106 g, 0.534 mmol, 1 eq) in DCM (5.34 mL) at rt. The reaction mixture was stirred for 2 h before the solvent was removed under reduced pressure to give crude product. The crude product was purified by flash column chromatography eluting with ethyl acetate:hexane (1:9) to give diester bicyclodihydropyridazine **58** as a colourless solid (0.111g, 74 %): $\mathbf{R_f}$ (EtOAc:hexane,

1:3) 0.30; **mp** 104-105 °C, literature 107 °C, H_2O:MeOH;[59] **IR** (neat, cm^{-1}) 3351 (N-H), 1722 (C=O), 1695 (C=O), 1255 (C-O); **^{1}H NMR δ** (500 MHz, $CDCl_3$) 8.58 (1H, br s, RN*H*N), 3.86 (3H, s, CO_2CH_3), 3.85 (3H, s, CO_2CH_3), 3.82-3.79 (1H, m, R_3CH), 3.45 (1H, ddd, *J* 16.0, 10.5 and 3.3 Hz, $RCH_{2ax}CR_2$), 2.10 (1H, ddd, *J* 16.0, 7.4 and 3.3 Hz, $RCH_{2eq}CR_2$), 2.00-1.91 (1H, m, RCH_2R), 1.75-1.56 (5H, m, RCH_2R), 1.45-1.34 (4H, m, RCH_2R); **^{13}C NMR δ** (125 MHz, $CDCl_3$) 164.7 (1C, RCO_2R), 162.5 (1C, RCO_2R), 132.7 (1C, $NCRCO_2R$), 128.7 (1C, $RCNHCO_2R$), 124.8 (1C, R_3C), 52.7 (1C, CO_2CH_3), 52.4 (1C, CO_2CH_3), 36.0 (1C, R_3CH), 31.4 (1C, RCH_2CR_2), 29.7 (1C, RCH_2R), 28.7 (1C, RCH_2R), 26.4 (1C, RCH_2R), 24.0 (1C, RCH_2R), 23.7 (1C, RCH_2R); ***m/z*** (ESI+, MeCN) 281.2 ($[M+H]^+$, 7 %), 303.2 ($[M+Na]^+$, 19 %), 583.2 ($[2M+Na]^+$, 100 %); **HRMS** (ESI+, MeCN) $[M+H]^+$ found 281.1492 ($C_{14}H_{21}N_2O_4$ requires 281.1496); **HPLC** (column 2, method A, ELSD, MeCN) t_R 7.38 min.

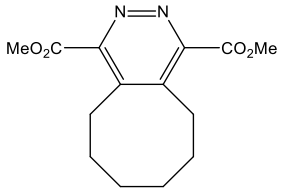
1,4-Dimethyl 5*H*,6*H*,7*H*,8*H*,9*H*,10*H*-Cycloocta[d]pyridazine-1,4-dicarboxylate 59



Cycloalkyne **50** (61.2 mg, 0.566 mmol, 1.05 eq) was added dropwise to a stirring solution of diester tetrazine **46** (0.107 g, 0.539 mmol, 1 eq) in DCM (5.39 mL) at rt. The reaction mixture was stirred for 2 h before the solvent was removed under reduced pressure to give crude product. The crude product was purified by flash column chromatography eluting with ethyl acetate:hexane (1:3) to give diester bicyclopyridazine **59** as a colourless solid (0.138 g, 92 %): **R_f** (EtOAc:hexane, 1:3) 0.26; **mp** 85-87 °C; **IR** (neat, cm^{-1}) 1723 (C=O), 1260 (C-O); **^{1}H NMR δ** (500 MHz, $CDCl_3$) 4.04 (6H, s, CO_2CH_3), 2.97-2.95 (4H, m, $ArCH_2R$), 1.86-1.85 (4H, m, RCH_2R), 1.43-1.38 (4H, m RCH_2R); **^{13}C NMR δ** (125 MHz, $CDCl_3$) 165.9 (2C, $ArCO_2R$), 153.6 (2C, $NCRCO_2R$), 141.6 (2C, R_3C), 53.3 (2C, CO_2CH_3), 30.5 (2C, RCH_2R), 27.0 (2C, $ArCH_2R$), 26.0 (2C, RCH_2R); ***m/z*** (ESI+, MeCN) 301.2 ($[M+Na]^+$, 58 %), 317.2 ($[M+K]^+$, 6 %); **HRMS** (ESI+, MeCN) $[M+H]^+$ found 279.1328

($C_{14}H_{19}N_2O_4$ requires 279.1339); **HPLC** (column 2, method A, ELSD, MeCN) t_R 6.00 min.

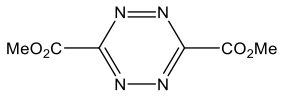
Optimisation of *in situ* Oxidation and Inverse Electron Demand Diels-Alder Reaction



Isopentyl nitrite was added to a stirring solution of diester dihydrotetrazine **45** (7.19 mg, 35.9 µmol, 1 eq) and cycloalkyne **50** (4.08 mg, 37.7 µmol, 1.05 eq) in DCM (0.359 mL) at rt. The reaction mixture was stirred for 2 h before the solvent was removed under reduced pressure to give crude product. The crude product was dissolved in deuterated chloroform (~0.75 mL) and a proton NMR spectrum taken immediately. Pseudoquantitative proton NMR yields were calculated from the ratio of the CO_2CH_3 peaks of diester tetrazine **46** and diester bicyclopyridazine **59** in the proton NMR spectrum.

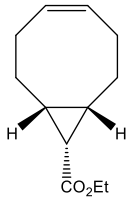
7.4. Chapter 4 Experimental

Investigation into the Oxidation of Dihydro-1,2,4,5-tetrazine



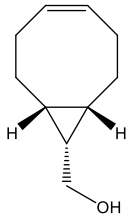
Oxidant (0.152 mmol, 3 eq) was added to a stirring solution of diester dihydrotetrazine **45** (10.1 mg, 50.5 µmol, 1 eq) and durene (3.40 mg, 25.3 µmol, 0.5 eq) in DCM-d_2 (0.505 mL) at 25 °C. The reaction mixture was stirred for 2 h before transferring into an NMR tube and a proton NMR spectrum taken immediately. qNMR yields were calculated from the Ar*H* peak of durene; and the CO_2CH_3 peaks of diester dihydrotetrazine **45** and diester tetrazine **46** in the proton NMR spectrum.

Ethyl (1*R*,4*Z*,8*S*,9*S*)-Bicyclo[6.1.0]non-4-ene-9-carboxylate 65[100]



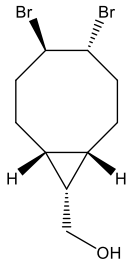
Ethyl diazoacetate (2.47 mL, 20.4 mmol, 1 eq) in anhydrous DCM (10 mL) was added dropwise to a stirring solution of *cis*,*cis*-1,5-cyclooctadiene (COD) (20.0 mL, 0.163 mol, 8 eq) and rhodium(II) acetate dimer (0.451 g, 1.02 mmol, 5 mol%) in anhydrous DCM (10 mL) at rt under nitrogen. The reaction mixture was stirred for 40 h before the solvent was removed under reduced pressure to give crude product. The crude product was purified by flash column chromatography eluting with hexane up to diethyl ether:hexane (1:99) to give (*Z*)-*endo*-bicycloalkene ester **65** as a colourless oil (1.19 g, 30 %): **R_f** (Et_2O:hexane, 1:99) 0.26; **IR** (neat, cm^{-1}) 1720 (C=O), 1152 (C-O); **^{1}H NMR δ** (500 MHz, $CDCl_3$) 5.63-5.57 (2H, m, RC*H*R), 4.11 (2H, q, *J* 7.2 Hz, CO_2CH_2R), 2.53-2.47 (2H, m, $RCHCH_2R$), 2.23-2.16 (2H, m, RCH_2CHR_2), 2.08-2.01 (2H, m, $RCHCH_2R$), 1.85-1.79 (2H, m, RCH_2CHR_2), 1.70 (1H, t, *J* 8.8 Hz, R_2CHCO_2R), 1.42-1.35 (2H, m, R_3CH), 1.26 (3H, t, *J* 7.2 Hz, RCH_3); **^{13}C NMR δ** (125 MHz, $CDCl_3$) 172.4 (1C, RCO_2R), 129.5 (2C, RCHR), 59.8 (1C, CO_2CH_2R), 27.2 (2C, $RCHCH_2R$), 24.3 (2C, R_3CH), 22.8 (2C, RCH_2CHR_2), 21.3 (1C, R_2CHCO_2R), 14.5 (1C, RCH_3); ***m/z*** (FAB, MeCN) 195.2 ($[M+H]^+$, 7 %), 109.1 (31), 55.0 (100); **HPLC** (column 2, method A, ELSD, MeCN) t_R not observed. The spectroscopic data was in agreement with the literature.[100] Further elution with diethyl ether:hexane (1:99) gave (*Z*)-*exo*-bicycloalkene ester **65** as a colourless oil (1.87 g, 47 %).

(1*R*,4*Z*,8*S*,9*S*)-Bicyclo[6.1.0]non-4-en-9-ylmethanol 66[100]



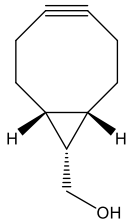
(*Z*)-*endo*-Bicycloalkene ester **65** (1.00 g, 5.15 mmol, 1 eq) in anhydrous diethyl ether (20 mL) was added dropwise to a stirring solution of lithium aluminium hydride (0.176 g, 4.64 mmol, 0.9 eq) in anhydrous diethyl ether (20 mL) at 0 °C under nitrogen. The reaction mixture was warmed to rt and stirred for 30 min before cooling to 0 °C. Water was added dropwise to the reaction mixture until the grey solid turned colourless. Anhydrous sodium sulfate was added to the reaction mixture and the insoluble solid removed by filtration. The filtrand was washed with diethyl ether (100 mL) before the solvent removed under reduced pressure to give crude product. The crude product was purified by flash column chromatography eluting with hexane up to ethyl acetate:hexane (1:4) to give (*Z*)-*endo*-bicycloalkene alcohol **66** as a colourless oil (0.778 g, 99 %): $\mathbf{R_f}$ (EtOAc:hexane, 1:4) 0.14; **IR** (neat, cm^{-1}) 3325 (O-H), 1673 (C=C); **^{1}H NMR δ** (500 MHz, $CDCl_3$) 5.65-5.59 (2H, m, RC*H*R), 3.70 (2H, d, *J* 7.5 Hz, RCH_2OH), 2.38-2.32 (2H, m, RCHCH_2R), 2.13-2.06 (2H, m, RCHCH_2R), 2.01-1.94 (2H, m, RCH_2CHR_2), 1.60-1.53 (2H, m, RCH_2CHR_2), 1.35 (1H, br s, RO*H*), 1.15-1.09 (1H, m, R_2C*H*CH_2OH), 1.04-0.96 (2H, m, R_3C*H*); **^{13}C NMR δ** (125 MHz, $CDCl_3$) 129.9 (2C, R*C*HR), 60.4 (1C, R*C*H_2OH), 27.8 (2C, RCH*C*H_2R), 24.0 (2C, R*C*H_2CHR_2), 20.8 (1C, R_2*C*HCH_2OH), 19.1 (2C, R_3*C*H); ***m/z*** (CI, MeCN) 153.1 ($[M+H]^+$, 52 %), 175.1 ($[M+Na]^+$, 18 %), 191.1 ($[M+K]^+$, 36 %), 167.1 (93), 165.1 (26), 154.0 (43), 153.1 (52), 152.1 (27), 151.1 (62), 149.1 (98), 147.1 (30), 137.1 (100); **HRMS** (CI, MeCN) $[M+H]^+$ found 153.1269 ($C_{10}H_{17}O_1$ requires 153.1274); **HPLC** (column 2, method A, ELSD, MeCN) t_R not observed.

(±)-[(1*R*,8*S*,9*S*)-*trans*-4,5-Dibromobicyclo[6.1.0]nonan-9-yl]methanol 67[100]



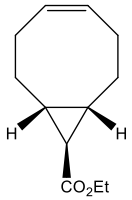
Bromine (0.254 mL, 4.95 mmol, 1.1 eq) in DCM (3.5 mL) was added dropwise to a stirring solution of (*Z*)-*endo*-bicycloalkene alcohol **66** (0.685 g, 4.50 mmol, 1 eq) in DCM (35 mL) at 0 °C. The reaction mixture was stirred for 30 min before warming to rt and diluting with sodium thiosulfate solution (10 mL, sat aq). The organic layer was separated from the aq layer and the aq layer extracted with DCM (3 × 20 mL). The organic layer and the combined extracts were dried over anhydrous sodium sulfate before the solvent was removed under reduced pressure to give crude product. The crude product was purified by flash column chromatography eluting with DCM to give (±)-*endo*,*trans*-dibromobicycloalkane alcohol **67** as a colourless solid (1.00 g, 71 %): **R_f** (DCM) 0.28; **mp** 116-118 °C; **IR** (neat, cm^{-1}) 3187 (O-H), 701 (C-Br); **^{1}H NMR δ** (500 MHz, $CDCl_3$) 4.82-4.78 (2H, m, RC*H*BrR), 3.79-3.71 (2H, m, RCH_2OH), 2.73-2.62 (2H, m, RCHBrCH_2R), 2.30-2.23 (1H, m, RCHBrCH_2R), 2.18-2.12 (1H, m, RCHBrCH_2R), 1.97-1.86 (2H, m, RCH_2CHR$_2$), 1.67-1.53 (2H, m, RCH_2CHR$_2$), 1.38 (1H, br s, RO*H*), 1.24-1.05 (3H, m, R$_2$C*H*CH$_2$OH and R$_3$C*H*); **^{13}C NMR δ** (125 MHz, $CDCl_3$) 59.7 (1C, R*C*H$_2$OH), 56.3 (1C, R*C*HBrR), 53.4 (1C, R*C*HBrR), 35.1 (2C, RCHBr*C*H$_2$R), 22.0 (1C, R$_2$*C*HCH$_2$OH), 20.2 (1C, R$_3$*C*H), 20.0 (1C, R*C*H$_2$CHR$_2$), 19.1 (1C, R*C*H$_2$CHR$_2$), 17.3 (1C, R$_3$*C*H); ***m/z*** (CI, MeCN) 310.9 ([M+H]$^+$, 23 %), 333.2 ([M+Na]$^+$, 4 %), 319.2 (33), 297.9 (100); **HRMS** (CI, MeCN) [M+H]$^+$ found 310.9636 ($Br_2C_{10}H_{17}O_1$ requires 310.9641); **HPLC** (column 2, method A, ELSD, MeCN) t_R 7.48 min.

(1*R*,8*S*,9*S*)-Bicyclo[6.1.0]non-4-yn-9-ylmethanol 60[100]



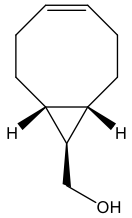
1 M Potassium *tert*-butoxide solution in anhydrous THF (10.6 mL, 10.6 mmol, 3.3 eq) was added dropwise to a stirring solution of (±)-*endo*,*trans*-dibromobicycloalkane alcohol **67** (1.00 g, 3.20 mmol, 1 eq) in anhydrous THF (35 mL) at 0 °C under nitrogen. The reaction mixture was heated to reflux and stirred for 2 h before cooling to rt and diluting with ammonium chloride solution (30 mL, sat aq). The aq layer was extracted with DCM (3 × 30 mL). The combined organic extracts were washed with water (1 × 30 mL) and sodium chloride solution (1 × 30 mL, sat aq) then dried over anhydrous sodium sulfate before the solvent was removed under reduced pressure to give crude product. The crude product was purified by flash column chromatography eluting with hexane up to ethyl acetate:hexane (3:17) to give *endo*-BCN-OH **60** as a colourless solid (0.175 g, 36 %): **R_f** (EtOAc:hexane, 3:17) 0.26; **mp** 58-60 °C; **IR** (neat, cm^{-1}) 3304 (O-H), 2184 (C≡C); **^{1}H NMR δ** (500 MHz, $CDCl_3$) 3.71 (2H, d, *J* 8.0 Hz, RC*H*$_2$OH), 2.31-2.18 (6H, m, RC*H*$_2$CHR$_2$ and RCC*H*$_2$R), 1.62-1.55 (2H, m, RC*H*$_2$CHR$_2$), 1.44 (1H, br s, RO*H*), 1.35-1.29 (1H, m, R$_2$C*H*CH$_2$OH), 0.94-0.90 (2H, m, R$_3$C*H*); **^{13}C NMR δ** (125 MHz, $CDCl_3$) 99.0 (2C, R*C*R), 60.0 (1C, R*C*H$_2$OH), 29.2 (2C, R*C*H$_2$CHR$_2$), 21.6 (2C, RC*C*H$_2$R), 21.5 (1C, R$_2$*C*HCH$_2$OH), 20.1 (2C, R$_3$*C*H); ***m/z*** (EI, MeCN) 150.1 ([M]$^{+\cdot}$, 13 %), 149.0 (100), 135.1 (61), 131.1 (68); **HPLC** (column 2, method A, ELSD, MeCN) t_R not observed. The spectroscopic data was in agreement with the literature.[100]

Ethyl (1*R*,4*Z*,8*S*,9*R*)-Bicyclo[6.1.0]non-4-ene-9-carboxylate 65[100]



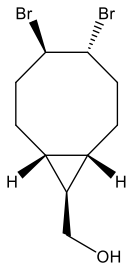
Ethyl diazoacetate (2.47 mL, 20.4 mmol, 1 eq) in anhydrous DCM (10 mL) was added dropwise to a stirring solution of COD (20.0 mL, 0.163 mol, 8 eq) and rhodium(II) acetate dimer (0.451 g, 1.02 mmol, 5 mol%) in anhydrous DCM (10 mL) at rt under nitrogen. The reaction mixture was stirred for 40 h before the solvent was removed under reduced pressure to give crude product. The crude product was purified by flash column chromatography eluting with hexane up to diethyl ether:hexane (1:99) to give (*Z*)-*endo*-bicycloalkene ester **65** as a colourless oil (1.19 g, 30 %). Further elution with diethyl ether:hexane (1:99) gave (*Z*)-*exo*-bicycloalkene ester **65** as a colourless oil (1.87 g, 47 %): **R$_f$** (Et_2O:hexane, 1:99) 0.23; **IR** (neat, cm^{-1}) 1719 (C=O), 1154 (C-O); **^{1}H NMR δ** (500 MHz, $CDCl_3$) 5.66-5.60 (2H, m, RC*H*R), 4.10 (2H, q, *J* 7.0 Hz, CO_2CH_2R), 2.33-2.27 (2H, m, $RCHCH_2R$), 2.22-2.16 (2H, m, RCH_2CHR_2), 2.11-2.05 (2H, m, $RCHCH_2R$), 1.59-1.53 (2H, m, R_3CH), 1.51-1.44 (2H, m, RCH_2CHR_2), 1.25 (3H, t, *J* 7.0 Hz, RCH_3), 1.18 (1H, t, *J* 4.5 Hz, R_2CHCO_2R); **^{13}C NMR δ** (125 MHz, $CDCl_3$) 174.6 (1C, RCO_2R), 130.1 (2C, RCHR), 60.4 (1C, CO_2CH_2R), 28.4 (2C, RCH_2CHR_2), 28.0 (1C, R_2CHCO_2R), 27.9 (2C, R_3CH), 26.8 (2C, $RCHCH_2R$), 14.4 (1C, RCH_3); ***m/z*** (FAB, MeCN) 195.1 ($[M+H]^+$, 12 %), 149.1 (36), 121.1 (93), 107.1 (33), 57.1 (100); **HPLC** (column 2, method A, ELSD, MeCN) t_R not observed. The spectroscopic data was in agreement with the literature.[100]

(1*R*,4*Z*,8*S*,9*R*)-Bicyclo[6.1.0]non-4-en-9-ylmethanol 66[100]



(*Z*)-*exo*-Bicycloalkene ester **65** (1.65 g, 8.49 mmol, 1 eq) in anhydrous diethyl ether (33 mL) was added dropwise to a stirring solution of lithium aluminium hydride (0.290 g, 7.64 mmol, 0.9 eq) in anhydrous diethyl ether (33 mL) at 0 °C under nitrogen. The reaction mixture was warmed to rt and stirred for 30 min before cooling to 0 °C. Water was added dropwise to the reaction mixture until the grey solid turned colourless. Anhydrous sodium sulfate was added to the reaction mixture and the insoluble solid removed by filtration. The filtrand was washed with diethyl ether (150 mL) before the solvent removed under reduced pressure to give crude product. The crude product was purified by flash column chromatography eluting with hexane up to ethyl acetate:hexane (1:4) to give (*Z*)-*exo*-bicycloalkene alcohol **66** as a colourless oil (1.10 g, 85 %): **R_f** (EtOAc:hexane 1:4) 0.13; **IR** (neat, cm^{-1}) 3317 (O-H); **^{1}H NMR δ** (500 MHz, $CDCl_3$) 5.66-5.60 (2H, m, RC*H*R), 3.48-3.46 (2H, d, *J* 7.0 Hz, R*C*H_2OH), 2.32-2.26 (2H, m, RCHCH_2R), 2.20-2.13 (2H, m, R*C*H_2CHR_2), 2.10-2.03 (2H, m, RCHCH_2R), 1.44-1.37 (3H, m, R*C*H_2CHR_2 and RO*H*), 0.81-0.74 (2H, m, R_3C*H*), 0.68-0.63 (1H, m, R_2C*H*CH_2OH); **^{13}C NMR δ** (125 MHz, $CDCl_3$) 130.3 (2C, R*C*HR), 67.4 (1C, R*C*H_2OH), 29.2 (2C, R*C*H_2CHR_2), 29.0 (1C, R_2*C*HCH_2OH), 27.2 (2C, RCH*C*H_2R), 22.2 (2C, R_3*C*H); ***m/z*** (CI, MeCN) 153.1 ($[M+H]^+$, 13 %), 152.1 (62), 151.1 (100); **HPLC** (column 2, method A, ELSD, MeCN) t_R not observed. The spectroscopic data was in agreement with the literature.[98]

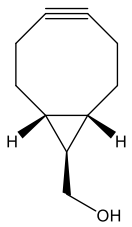
(±)-[(1*R*,8*S*,9*R*)-*trans*-4,5-Dibromobicyclo[6.1.0]nonan-9-yl]methanol **67**[100]



Bromine (0.374 mL, 7.23 mmol, 1.1 eq) in DCM (5 mL) was added dropwise to a stirring solution of (*Z*)-*exo*-bicycloalkene alcohol **66** (1.00 g, 6.57 mmol, 1 eq) in DCM (50 mL) at 0 °C. The reaction mixture was stirred for 30 min before warming to rt and

diluting with sodium thiosulfate solution (10 mL, sat aq). The organic layer was separated from the aq layer and the aq layer extracted with DCM (3 × 20 mL). The organic layer and the combined extracts were dried over anhydrous sodium sulfate before the solvent was removed under reduced pressure to give crude product. The crude product was purified by flash column chromatography eluting with DCM to give (±)-*exo*,*trans*-dibromobicycloalkane alcohol **67** as a colourless solid (1.74 g, 85 %): **R_f** (DCM) 0.16; **mp** 49-51 °C; **IR** (neat, cm^{-1}) 3261 (O-H), 1024 (C-O), 699 (C-Br); **^{1}H NMR δ** (400 MHz, $CDCl_3$) 4.84-4.77 (2H, m, RC*H*BrR), 3.49 (2H, d, *J* 12.0 Hz, RCH_2OH), 2.69-2.55 (2H, m, RCHBrCH_2R), 2.28-2.19 (1H, m, RCHBrCH_2R), 2.10-2.03 (3H, m, RCHBrCH_2R and RCH_2CHR$_2$), 1.71 (1H, s, RO*H*), 1.48-1.30 (2H, m, RCH_2CHR$_2$), 0.94-0.80 (2H, m, R$_3$C*H*), 0.68-0.62 (1H, m, R$_2$C*H*CH$_2$OH); **^{13}C NMR δ** (100 MHz, $CDCl_3$) 66.6 (1C, R*C*H$_2$OH), 56.3 (1C, R*C*HBrR), 53.4 (1C, R*C*HBrR), 35.0 (1C, RCHBr*C*H$_2$R), 34.9 (1C, RCHBr*C*H$_2$R), 28.2 (1C, R$_2$*C*HCH$_2$OH), 24.5 (1C, R*C*H$_2$CHR$_2$), 23.7 (1C, R*C*H$_2$CHR$_2$), 22.6 (1C, R$_3$*C*H), 19.9 (1C, R$_3$*C*H); ***m/z*** (CI, MeCN) 310.9 ([M+H]$^+$, 100 %), 308.9 (56); **HRMS** (CI, MeCN) [M+H]$^+$ found 310.9469 ($Br_2C_{10}H_{17}O_1$ requires 310.9641); **HPLC** (column 2, method A, ELSD, MeCN) t_R 6.90 min.

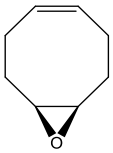
(1*R*,8*S*,9*R*)-Bicyclo[6.1.0]non-4-yn-9-ylmethanol 60[100]



1 M Potassium *tert*-butoxide solution in anhydrous THF (16.6 mL, 16.6 mmol, 3.3 eq) was added dropwise to a stirring solution of (±)-*exo*,*trans*-dibromobicycloalkane alcohol **67** (1.57 g, 5.03 mmol, 1 eq) in anhydrous THF (55 mL) at 0 °C under nitrogen. The reaction mixture was heated to reflux and stirred for 2 h before cooling to rt and diluting with ammonium chloride solution (50 mL, sat aq). The aq layer was extracted with DCM (3 × 50 mL). The combined organic extracts were washed with water (1 × 50 mL) and sodium chloride solution (1 × 50 mL, sat aq) then dried over anhydrous

sodium sulfate before the solvent was removed under reduced pressure to give crude product. The crude product was purified by flash column chromatography eluting with hexane up to ethyl acetate:hexane (3:17) to give *exo*-BCN-OH **60** as a colourless solid (0.246 g, 33 %): **R_f** (EtOAc:hexane, 3:17) 0.16; **mp** 60-61 °C; **IR** (neat, cm^{-1}) 3264 (O-H); **^{1}H NMR δ** (500 MHz, $CDCl_3$) 3.55 (2H, d, *J* 6.2 Hz, RC*H*$_2$OH), 2.43-2.40 (2H, m, RC*H*$_2$CHR$_2$), 2.31-2.26 (2H, m, RCC*H*$_2$R), 2.17-2.14 (2H, m, RCC*H*$_2$R), 1.42-1.35 (2H, m, RC*H*$_2$CHR$_2$), 0.73-0.64 (3H, m, R$_3$C*H* and R$_2$C*H*CH$_2$OH); **^{13}C NMR δ** (125 MHz, $CDCl_3$) 99.0 (2C, R*C*R), 67.3 (1C, R*C*H$_2$OH), 33.6 (2C, RCH$_2$CHR$_2$), 27.5 (1C, R$_2$*C*HCH$_2$OH), 22.7 (2C, R$_3$*C*H), 21.6 (2C, RC*C*H$_2$R); ***m/z*** (CI, MeCN) 151.1 ($[M+H]^+$, 24 %), 135.1 (25), 42.4 (100); **HPLC** (column 2, method A, ELSD, MeCN) t_R not observed. The spectroscopic data was in agreement with the literature.[100]

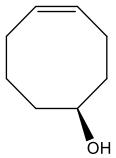
(4*Z*)-9-Oxabicyclo[6.1.0]non-4-ene 68[125]



*m*CPBA (50.0 g, 0.203 mol, 1 eq) in chloroform (460 mL) was added dropwise to a stirring solution of COD (22.0 g, 0.203 mol, 1 eq) at rt. The reaction mixture was stirred overnight before the insoluble solid was removed by filtration. The filtrate was washed with sodium bisulfate solution (1 × 450 mL, sat aq), sodium bicarbonate solution (1 × 450 mL, sat aq) and sodium chloride solution (1 × 450 mL, sat aq) then dried over anhydrous magnesium sulfate before the solvent was removed under reduced pressure to give crude product. The crude product was purified by flash column chromatography eluting with ethyl acetate:hexane (1:19) to give (*Z*)-cycloalkene epoxide **68** as a colourless oil (14.0 g, 56 %): **R_f** (EtOAc:hexane, 1:19) 0.25; **IR** (neat, cm^{-1}) 1656 (C=C); **^{1}H NMR δ** (500 MHz, $CDCl_3$) 5.60-5.52 (2H, m, RC*H*R), 3.05-3.01 (2H, m, RC*H*OR), 2.47-2.41 (2H, m, RCHC*H*$_2$R), 2.17-2.09 (2H, m, RC*H*$_2$CHOR), 2.07-1.98 (4H, m, RCHC*H*$_2$R and RC*H*$_2$CHOR); **^{13}C NMR δ** (125 MHz, $CDCl_3$) 129.0 (2C, R*C*HR), 56.8 (2C, R*C*HOR), 28.2 (2C, R*C*H$_2$CHOR), 23.8

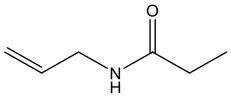
(2C, RCH*C*H$_2$R); ***m/z*** (CI, MeCN) 125.1 ([M+H]$^+$, 12 %), 124.1 (36), 108.1 (26), 107.1 (100); **HPLC** (column 2, method A, ELSD, MeCN) t_R not observed. The spectroscopic data was in agreement with the literature.[138]

(±)-(4*Z*)-Cyclooct-4-en-1-ol 61[125]



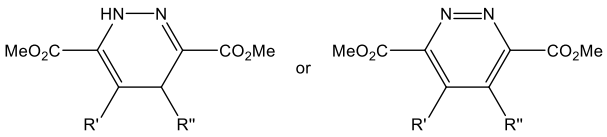
1 M Lithium aluminium hydride solution in anhydrous THF (53.5 mL, 53.5 mmol, 0.5 eq) was added dropwise to a stirring solution of (*Z*)-cycloalkene epoxide **68** (13.3 g, 0.107 mol, 1 eq) in anhydrous THF at rt under nitrogen. The reaction mixture was stirred overnight before cooling to 0 °C and diluting with water (50 mL). The insoluble solid was removed by filtration and washed with diethyl ether (3 × 20 mL) before the solvent was removed under reduced pressure to give crude product. The crude product was purified by flash column chromatography eluting with hexane up to ethyl acetate:hexane (3:7) to give (±)-(*Z*)-cycloalkene alcohol **61** as a colourless oil (9.78 g, 72 %): **R$_f$** (EtOAc:hexane, 3:7) 0.35; **IR** (neat, cm^{-1}) 3332 (O-H); **^{1}H NMR δ** (500 MHz, $CDCl_3$) 5.71-5.65 (1H, m, RC*H*R), 5.60-5.55 (1H, m, RC*H*R), 3.81-3.77 (1H, m, RC*H*OHR), 2.32-2.24 (1H, m, RCHC*H*$_2$R), 2.15-2.06 (3H, m, RCHC*H*$_2$R), 1.94-1.87 (1H, m, RC*H*$_2$CHOHR), 1.86-1.80 (1H, m, RC*H*$_2$CHOHR), 1.73-1.68 (1H, m, RC*H*$_2$CHOHR), 1.66-1.59 (2H, m, RC*H*$_2$R and RO*H*), 1.56-1.46 (2H, m, RC*H*$_2$CHOHR and RC*H*$_2$R); **^{13}C NMR δ** (125 MHz, $CDCl_3$) 130.3 (1C, R*C*HR), 129.7 (1C, R*C*HR), 72.8 (1C, R*C*HOHR), 37.8 (1C, R*C*H$_2$CHOHR), 36.4 (1C, R*C*H$_2$CHOHR), 25.8 (1C, RCH*C*H$_2$R), 25.0 (1C, R*C*H$_2$R), 22.9 (1C, RCH*C*H$_2$R); ***m/z*** (EI, MeCN) 126.1 ([M]$^{+\cdot}$, 8 %), 108.1 (32), 98.1 (100), 67.1 (100); **HPLC** (column 2, method A, ELSD, MeCN) t_R not observed. The spectroscopic data was in agreement with the literature.[125]

N-(Prop-2-en-1-yl)propanamide 62



Propionyl chloride (1.59 mL, 18.2 mmol, 1 eq) and triethylamine (3.04 mL, 21.8 mmol, 1.2 eq) were added to a stirring solution of allylamine (1.37 mL, 18.2 mmol, 1 eq) in DCM at 0 °C. The reaction mixture was warmed to rt and stirred for 2 h before diluting with hydrochloric acid (100 mL, 1 M aq). The organic layer was separated from the aq layer and the aq layer extracted with chloroform (3 × 50 mL). The organic layer and combined organic extracts were dried over magnesium sulfate before the solvent was removed under reduced pressure to give crude product. The crude product was purified by flash column chromatography eluting with ethyl acetate:hexane (1:1) to give allyl amide **62** as a yellow oil (1.92 g, 93 %): **R_f** (EtOAc:hexane, 1:1) 0.32; **IR** (neat, cm^{-1}) 1643 (C=O), 1546 (N-H); **^{1}H NMR δ** (400 MHz, $CDCl_3$) 5.83 (1H, ddt, *J* 17.2, 10.4 and 5.6 Hz, $H_2CC\mathit{H}R$), 5.60 (1H, br s, $RN\mathit{H}COR$), 5.17 (1H, dq, *J* 17.2 and 1.6 Hz, H_2CCHR), 5.12 (1H, dq, *J* 10.4 and 1.6 Hz, H_2CCHR), 3.88 (2H, tt, *J* 5.6 and 1.6 Hz, $H_2CCHC\mathit{H}_2R$), 2.23 (2H, q, *J* 7.6 Hz, $NHCOC\mathit{H}_2R$), 1.16 (3H, t, *J* 7.6 Hz, $RC\mathit{H}_3$); **^{13}C NMR δ** (125 MHz, $CDCl_3$) 173.7 (1C, $RNHC\mathit{C}OR$), 134.4 (1C, $H_2C\mathit{C}HR$), 116.5 (1C, $H_2\mathit{C}CHR$), 42.0 (1C, $H_2CCH\mathit{C}H_2R$), 29.8 (1C, $NHCO\mathit{C}H_2R$), 10.0 (1C, $R\mathit{C}H_3$); ***m/z*** (ESI+, MeCN) 114.3 ($[M+H]^+$, 76 %), 136.2 ($[M+Na]^+$, 6 %), 227.3 ($[2M+H]^+$, 100 %), 249.2 ($[2M+Na]^+$, 13 %); **HRMS** (EI, MeCN) $[M]^{+\cdot}$ found 113.0833 ($C_6H_{11}NO$ requires 113.0835); **HPLC** (column 1, method A, ELSD, MeCN) t_R not observed. The spectroscopic data was in agreement with the literature.[139]

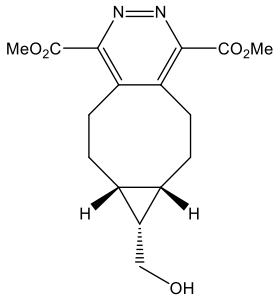
Inverse Electron Demand Diels-Alder Reactions



Dienophile (53.0 µmol, 1.05 eq) was added to a stirring solution of diester tetrazine **46** (10.0 mg, 50.5 µmol, 1 eq) and durene (3.40 mg, 25.3 µmol, 0.5 eq) in DCM-d_2 (0.505

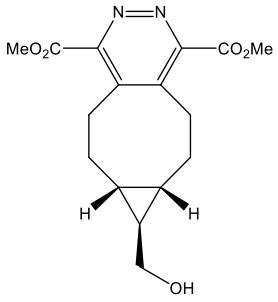
mL) at 25 °C. The reaction mixture was stirred for 2 h before transferring to an NMR tube and a proton NMR spectrum taken immediately. The qNMR yield was calculated from the Ar*H* peak of durene and a characteristic peak of diester (dihydro)pyridazine in the proton NMR spectrum.

10,13-Dimethyl (4*R*,5*S*,6*S*)-5-(Hydroxymethyl)-11,12-diazatricyclo[7.4.0.0^{4,6}]trideca-1(13),9,11-triene-10,13-dicarboxylate 70



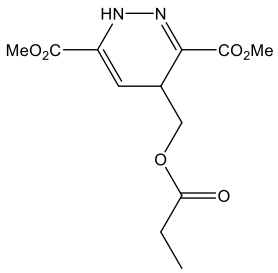
endo-BCN-OH **60** (49.3 mg, 0.328 mmol, 1.05 eq) was added slowly to a stirring solution of diester tetrazine **46** (61.8 mg, 0.312 mmol, 1 eq) in DCM (3.12 mL) at 25 °C. The reaction mixture was stirred for 2 h before the solvent was removed under reduced pressure to give crude product. The crude product was purified by automated flash column chromatography eluting with DCM to give *endo*-diester tricyclopyridazine alcohol **70** as a colourless solid (85.0 mg, 85 %): **R$_f$** (DCM:MeOH, 39:1) 0.32; **mp** 124-126 °C; **IR** (neat, cm^{-1}) 3411 (O-H), 1730 (C=O), 1147 (C-O); **^{1}H NMR δ** (400 MHz, $CDCl_3$) 4.03 (6H, s, CO_2CH_3), 3.70 (2H, d, *J* 7.7 Hz, RCH_2OH), 3.08-2.94 (4H, m, $ArCH_2R$), 2.44-2.39 (2H, m, RCH_2CHR_2), 1.65-1.53 (3H, m, RCH_2CHR_2 and ROH), 1.17-1.08 (1H, m, R_2CHCH_2OH), 0.97-0.87 (2H, m, R_3CH); **^{13}C NMR δ** (100 MHz, $CDCl_3$) 166.0 (2C, $ArCO_2R$), 154.2 (2C, $NCRCO_2R$), 143.3 (2C, R_3C), 59.4 (1C, RCH_2OH), 53.4 (2C, CO_2CH_3), 27.7 (2C, $ArCH_2R$), 23.6 (2C, RCH_2CHR_2), 22.4 (1C, R_2CHCH_2OH), 18.8 (2C, R_3CH); ***m/z*** (ESI+, MeCN) 321.0 ($[M+H]^+$, 14 %), 662.9 ($[2M+Na]^+$, 30 %), 146.0 (100), 105.2 (64); **HRMS** (ESI+, MeCN) $[M+H]^+$ found 321.1452 ($C_{16}H_{21}N_2O_5$ requires 321.1445); **HPLC** (column 2, method A, ELSD, MeCN) t_R 4.35 min.

10,13-Dimethyl (4*R*,5*R*,6*S*)-5-(Hydroxymethyl)-11,12-diazatricyclo[7.4.0.0^{4,6}]trideca-1(13),9,11-triene-10,13-dicarboxylate 70



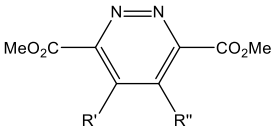
exo-BCN-OH **60** (49.3 mg, 0.328 mmol, 1.05 eq) was added slowly to a stirring solution of diester tetrazine **46** (61.8 mg, 0.312 mmol, 1 eq) in DCM (3.12 mL) at 25 °C. The reaction mixture was stirred for 2 h before the solvent was removed under reduced pressure to give crude product. The crude product was purified by automated flash column chromatography eluting with DCM up to DCM:methanol (39:1) to give *exo*-diester tricyclopyridazine alcohol **70** as a colourless solid (89.0 mg, 89 %): **R$_f$** (DCM:MeOH, 39:1) 0.29; **mp** 107-109 °C; **IR** (neat, cm^{-1}) 3414 (O-H), 1728 (C=O), 1160 (C-O); **^{1}H NMR δ** (400 MHz, $CDCl_3$) 4.01 (6H, s, CO_2CH_3), 3.34 (2H, d, *J* 6.8 Hz, RCH_2OH), 3.03-2.89 (4H, m, $ArCH_2R$), 2.63-2.54 (2H, m, RCH_2CHR_2), 1.80 (1H, br s, RO*H*), 1.48-1.39 (2H, m, RCH_2CHR_2), 0.80-0.74 (1H, m, R_2CHCH_2OH), 0.68-0.60 (2H, m, R_3CH); **^{13}C NMR δ** (100 MHz, $CDCl_3$) 165.9 (2C, $ArCO_2R$), 154.1 (2C, $NCRCO_2R$), 142.5 (2C, R_3C), 65.9 (1C, RCH_2OH), 53.3 (2C, CO_2CH_3), 30.4 (1C, R_2CHCH_2OH), 28.5 (2C, RCH_2CHR_2), 27.4 (2C, $ArCH_2R$), 20.8 (2C, R_3CH); ***m/z*** (ESI+, MeCN) 321.0 ([M+H]$^+$, 18 %), 342.9 ([M+Na]$^+$, 6 %), 662.9 ([2M+Na]$^+$, 51 %), 146.1 (100), 105.2 (70); **HRMS** (ESI+, MeCN) [M+H]$^+$ found 321.1446 ($C_{16}H_{21}N_2O_5$ requires 321.1445); **HPLC** (column 2, method A, ELSD, MeCN) t_R 4.41 min.

3,6-Dimethyl 4-[(Propanoyloxy)methyl]-1,4-dihydropyridazine-3,6-dicarboxylate 73



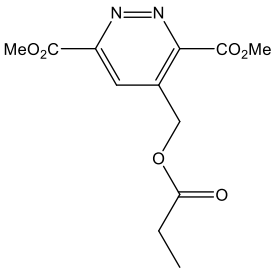
Allyl propionate (46.2 µl, 0.370 mmol, 1.05 eq) was added slowly to a stirring solution of diester tetrazine **46** (69.7 mg, 0.352 mmol, 1 eq) in DCM (3.52 mL) at 25 °C. The reaction mixture was stirred for 2 h before the solvent was removed under reduced pressure to give crude product. The crude product was purified by flash column chromatography eluting with DCM to give diester dihydropyridazine ester **73** as a colourless gum (84.0 mg, 84 %): **R_f** (DCM:MeOH, 99:1) 0.24; **IR** (neat, cm^{-1}) 3352 (N-H), 1726 (C=O), 1173 (C-O), 1122 (C-O); **^{1}H NMR δ** (500 MHz, $CDCl_3$) 8.33 (1H, br s, RN*H*N), 5.84-5.83 (1H, m, RC*H*R), 4.15 (1H, dd, *J* 10.8 and 5.0 Hz, RCH_2OCOR), 4.03-4.00 (1H, m, R_3CH), 3.94 (1H, dd, *J* 10.8 and 5.0 Hz, RCH_2OCOR), 3.87 (3H, s, CO_2CH_3), 3.86 (3H, s, CO_2CH_3), 2.29 (2H, q, *J* 7.6 Hz, OCOCH_2R), 1.10 (3H, t, *J* 7.6 Hz, RCH_3); **^{13}C NMR δ** (125 MHz, $CDCl_3$) 174.3 (1C, RO*C*OR), 164.6 (1C, R*C*O_2R), 161.5 (1C, R*C*O_2R), 130.7 (1C, N*C*RCO_2R), 130.6 (1C, R*C*NHCO_2R), 106.4 (1C, R*C*HR), 64.5 (1C, RCH$_2$OCOR), 52.9 (1C, CO_2CH_3), 52.8 (1C, CO_2CH_3), 32.2 (1C, R_3CH), 27.6 (1C, OCO*C*H_2R), 9.1 (1C, R*C*H_3); ***m/z*** (ESI+, MeCN) 285.0 ($[M+H]^+$, 5 %), 307.0 ($[M+Na]^+$, 45 %), 322.9 ($[M+K]^+$, 10 %), 590.8 ($[2M+Na]^+$, 100 %), 146.0 (83), 105.2 (69); **HRMS** (ESI+, MeCN) $[M+Na]^+$ found 307.0910 ($C_{12}H_{16}N_2NaO_6$ requires 307.0901); **HPLC** (column 2, method A, ELSD, MeCN) t_R 5.06 min.

Investigation into the *in situ* Oxidation and Inverse Electron Demand Diels-Alder Reactions



Oxidant (0.152 mmol, 3 eq) was added to a stirring solution of diester dihydrotetrazine **45** (10.1 mg, 50.5 µmol, 1 eq), durene (3.40 mg, 25.3 µmol, 0.5 eq) and dienophile (53.0 µmol, 1.05 eq) in DCM-d_2 (0.505 mL) at 25 °C. The reaction mixture was stirred for 2 h before transferring to an NMR tube and a proton NMR spectrum taken immediately. qNMR yields were calculated from the Ar*H* peak of durene; and the CO_2CH_3 peaks of diester tetrazine **46** and diester pyridazine in the proton NMR spectrum.

3,6-Dimethyl 4-[(Propanoyloxy)methyl]pyridazine-3,6-dicarboxylate 74

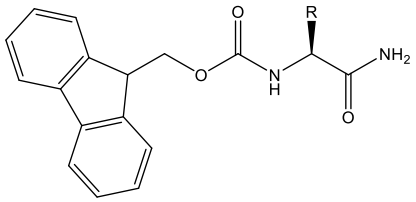


DDQ (0.241 g, 1.06 mmol, 3 eq) was added to a stirring solution of diester dihydrotetrazine **45** (70.9 mg, 0.354 mmol, 1 eq) and allyl propionate (46.5 µl, 0.372 mmol, 1.05 eq) in DCM at 25 °C. The reaction mixture was stirred for 2 h before the insoluble solid was removed by filtration and the solvent removed under reduced pressure to give crude product. The crude product was purified by flash column chromatography eluting with DCM to give diester pyridazine ester **74** as a yellow oil (97.4 mg, 97 %): **R_f** (DCM:MeOH, 99:1) 0.14; **IR** (neat, cm^{-1}) 1729 (C=O), 1263 (C-O), 1137 (C-O); **^{1}H NMR δ** (500 MHz, $CDCl_3$) 8.31 (1H, s, Ar*H*), 5.55 (2H, s, ArCH_2OCOR), 4.11 (3H, s, CO_2CH_3), 4.09 (3H, s, CO_2CH_3), 2.52 (2H, q, *J* 7.6 Hz,

OCOCH_2R), 1.23 (3H, t, *J* 7.6 Hz, RCH_3); **^{13}C NMR δ** (125 MHz, $CDCl_3$) 173.6 (1C, RO*C*OR), 164.6 (1C, Ar*C*O_2R), 164.1 (1C, Ar*C*O_2R), 152.4 (1C, Ar), 150.6 (1C, Ar), 139.8 (1C, Ar), 125.9 (1C, ArH), 61.2 (1C, Ar*C*H_2OCOR), 53.8 (2C, CO_2*C*H_3), 27.5 (1C, OCO*C*H_2R), 9.2 (1C, R*C*H_3); ***m/z*** (ESI+, MeCN) 283.0 ($[M+H]^+$, 14 %), 304.8 ($[M+Na]^+$, 8 %), 586.8 ($[2M+Na]^+$, 100 %), 130.3 (72); **HRMS** (ESI+, MeCN) $[M+Na]^+$ found 305.0745 ($C_{12}H_{14}N_2NaO_6$ requires 305.0744); **HPLC** (column 2, method A, MeCN, ELSD) t_R 5.15 min.

7.5. Chapter 5 Experimental

Stability of Resin-Bound Amino Acids towards Oxidant

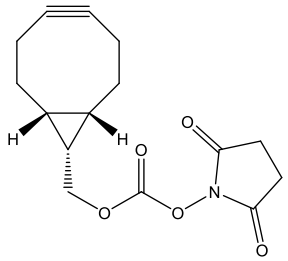


Aminomethyl polystyrene resin (25.0 mg, 0.745 $mmolg^{-1}$) was functionalised according to general procedure 1 then Fmoc deprotected and amino acid coupled with Fmoc-Aaa-OH (Table 6) according to general procedure 2. DDQ (12.7 mg, 55.8 µmol, 3 eq) was added to the resin in DCM (0.186 mL) at rt. The resin was agitated for 4 h then filtered before washing with DMF (× 3), DCM (× 3), methanol (× 3) and diethyl ether (× 3). The linker was cleaved according to general procedure 3 to give Fmoc-Aaa-NH_2 **79** which was analysed by analytical HPLC and LRMS (Table 6).

Entry	Fmoc-Aaa-OH	Fmoc-Aaa-NH_2 79	HPLC[a]		*m/z*[b]
			t_R/min	Purity/%	$[M+H]^+$
I	Fmoc-Arg(Pbf)-OH	Fmoc-Arg-NH_2	5.29	100	396.0 (100)
II	Fmoc-Asn(Trt)-OH	Fmoc-Asn-NH_2	6.05	100	354.0 (45)
III	Fmoc-Asp(O^{t}Bu)-OH	Fmoc-Asp-NH_2	6.38	100	355.0 (70)
IV	Fmoc-Cys(Trt)-OH	Fmoc-Cys-NH_2	7.30	100	343.0 (10)
V	Fmoc-Gln(Trt)-OH	Fmoc-Gln-NH_2	6.04	100	368.0 (75)
VI	Fmoc-Glu(O^{t}Bu)-OH	Fmoc-Glu-NH_2	6.37	100	368.9 (49)
VII	Fmoc-His(Trt)-OH	Fmoc-His-NH_2	4.96	100	377.0 (100)
VIII	Fmoc-Lys(Boc)-OH	Fmoc-Lys-NH_2	4.96	100	368.2 (100)
IX	Fmoc-Met-OH	Fmoc-Met-NH_2	6.13	96	370.9 (7)
X	Fmoc-Ser(^{t}Bu)-OH	Fmoc-Ser-NH_2	6.22	100	327.0 (24)
XI	Fmoc-Thr(^{t}Bu)-OH	Fmoc-Thr-NH_2	6.48	100	341.0 (53)
XII	Fmoc-Trp(Boc)-OH	Fmoc-Trp-NH_2	7.83	100	425.9 (31)
XIII	Fmoc-Tyr(^{t}Bu)-OH	Fmoc-Tyr-NH_2	6.93	100	403.0 (70)

Table 6. Investigation into the stability of resin-bound amino acids towards DDQ. [a] Column 2, method A, ELSD, H_2O:MeCN (1:1). [b] ESI+, H_2O:MeCN (1:1).

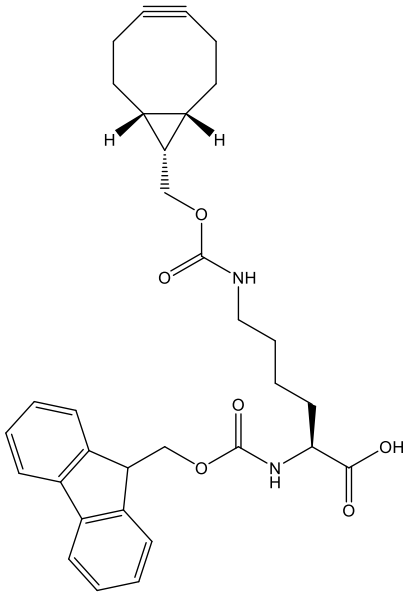
(1*R*,8*S*,9*S*)-Bicyclo[6.1.0]non-4-yn-9-ylmethyl 2,5-dioxopyrrolidin-1-yl carbonate 80[103]



DSC (0.256 g, 0.999 mmol, 1.5 eq) was added to a stirring solution of *endo*-BCN-OH **60** (0.100 g, 0.666 mmol, 1 eq) and triethylamine (0.371 mL, 2.66 mmol, 4 eq) in anhydrous acetonitrile at 0 °C under nitrogen. The reaction mixture was warmed to rt and stirred for four h before the solvent was removed under reduced pressure to give crude product. The crude product was purified by flash column chromatography eluting with hexane up to ethyl acetate:hexane (2:3) to give *endo*-BCN-OSu **80** as a colourless solid (0.139 g, 72 %): **R_f** (EtOAc:hexane, 2:3) 0.36; **mp** 119-121 °C; **IR** (neat, cm^{-1}) 1808 (C=O), 1788 (C=O), 1731 (C=O), 1212 (C-O), 1204 (C-O); **^{1}H NMR δ** (500 MHz, $CDCl_3$) 4.45 (2H, d, *J* 8.4 Hz, RC*H*$_2$OCO$_2$R), 2.83 (4H, s, R$_2$NCOC*H*$_2$R),

2.34-2.21 (6H, m, RCCH_2R and RCH_2CHR$_2$), 1.62-1.46 (3H, m, RCCH_2R and R$_2$CHCH$_2$OCO$_2$R), 1.09-1.02 (2H, m, R$_3$CH); **^{13}C NMR δ** (125 MHz, CDCl$_3$) 168.8 (2C, RCONR$_2$), 151.8 (1C, ROCO$_2$R), 98.8 (2C, RCR), 70.5 (1C, RCH$_2$OCO$_2$R), 29.1 (2C, RCCH$_2$R), 25.6 (2C, R$_2$NCOCH$_2$R), 21.5 (2C, R$_3$$C$H), 20.9 (2C, R$CH_2CHR_2$), 17.3 (1C, R$_2$$CHCH_2OCO_2$R); ***m/z*** (CI, MeCN) 292.3 ([M+H]$^+$, 27 %), 291.2 (100), 290.2 (81), 120.1 (100); **HRMS** (CI, MeCN) [M+H]$^+$ found 292.1181 (C$_{15}$H$_{18}$NO$_5$ requires 292.1180); **HPLC** (column 2, method A, ELSD, MeCN) t_R 7.99 min.

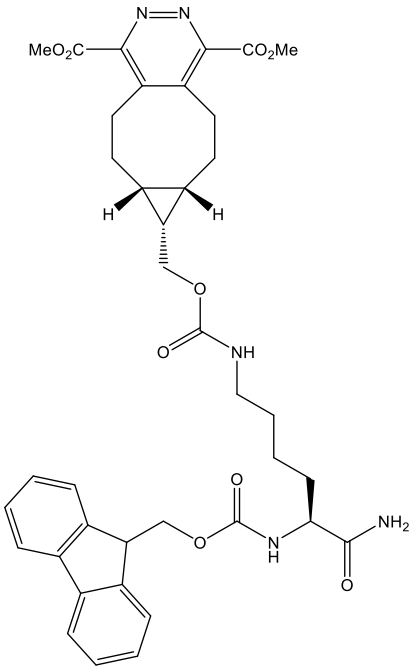
(2*S*)-6-({[(1*R*,8*S*,9*S*)-Bicyclo[6.1.0]non-4-yn-9-ylmethoxy]carbonyl} amino)-2-{[(9*H*-fluoren-9-ylmethoxy)carbonyl]amino}hexanoic acid 75[103]



endo-BCN-OSu **80** (0.200 g, 0.687 mmol, 1 eq) in anhydrous DMF (0.60 mL) was added to a stirring solution of Fmoc-Lys-OH hydrochloride (0.417 g, 1.03 mmol, 1.5 eq) and DIPEA (0.239 mL, 1.37 mmol, 2 eq) in anhydrous DMF (1.80 mL) at rt under nitrogen. The reaction mixture was stirred overnight before diluting with diethyl ether (20 mL). The reaction mixture was washed with water (3 × 20 mL) then dried over anhydrous magnesium sulfate before the solvent was removed under reduced pressure to give crude product. The crude product was purified by flash column chromatography eluting with DCM up to DCM:methanol (2:23) to give Fmoc-Lys(*endo*-BCN)-OH **75** as a colourless solid (0.200 g, 53 %): **R$_f$** (DCM:MeOH, 9:1)

0.33; **mp** 178-179 °C; **IR** (neat, cm^{-1}) 3314 (O-H), 1694 (C=O), 1242 (C-O); **^{1}H NMR δ** (500 MHz, DMSO-d_6) 7.87 (2H, d, *J* 7.5 Hz, Ar*H*), 7.70 (2H, d, *J* 6.0 Hz, Ar*H*), 7.40 (2H, t, *J* 7.5 Hz, Ar*H*), 7.32 (2H, t, *J* 7.5 Hz, Ar*H*), 7.04 (2H, br s, ROCON*H*R), 4.30-4.21 (3H, m, R_3C*H* and $RCH_2OCONHR$), 3.99 (2H, d, *J* 7.7 Hz, $RCH_2OCONHR$), 3.84 (1H, br s, ROCONHC*H*RCO_2H), 2.94 (2H, br s, ROCONHCH_2R), 2.22-2.10 (5H, m, RCCH_2R and RCH_2R), 1.71-1.21 (10H, m, RCH_2CHR_2, $R_2CHCH_2OCONHR$ and RCH_2R), 0.83-0.80 (2H, m, R_3CH); **^{13}C NMR δ** (125 MHz, DMSO-d_6) 175.6 (1C, RO*C*ONHR), 156.4 (1C, R*C*O_2H), 155.7 (1C, RO*C*ONHR), 144.0 (1C, Ar), 143.8 (1C, Ar), 140.7 (2C, Ar), 127.6 (2C, ArH), 127.0 (2C, ArH), 125.3 (1C, ArH), 125.2 (1C, ArH), 120.1 (2C, ArH), 99.0 (2C, R*C*R), 65.4 (1C, R*C*$H_2OCONHR$), 61.2 (1C, R*C*$H_2OCONHR$), 54.9 (1C, ROCONH*C*$HRCO_2H$), 46.8 (1C, R_3CH), 40.2 (1C, ROCONH*C*H_2R), 31.7 (1C, R*C*H_2R), 29.3 (2C, R*C*H_2CHR_2), 28.6 (1C, R*C*H_2R), 22.7 (1C, R*C*H_2R), 20.8 (2C, RC*C*H_2R), 19.5 (2C, R_3CH), 17.7 (1C, $R_2CHCH_2OCONHR$); ***m/z*** (ESI+, MeCN) 567.2 ($[M+Na]^+$, 25 %), 324.2 (27), 146.1 (50), 115.2 (30), 105.2 (52), 102.3 (100); **HRMS** (ESI+, MeCN) $[M+Na]^+$ found 567.2481 ($C_{32}H_{36}N_2O_6Na$ requires 567.2466); **HPLC** (column 3, method A, ELSD, MeCN) t_R 9.41 min.

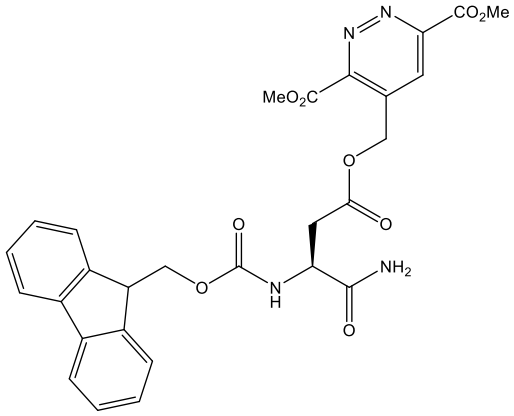
10,13-Dimethyl (4*R*,5*R*,6*S*)-5-[({[(5*S*)-5-carbamoyl-5-{[(9*H*-fluoren-9-ylmethoxy)carbonyl]amino}pentyl]carbamoyl}oxy)methyl]-11,12-diazatricyclo[7.4.0.0^{4,6}]trideca-1(13),9,11-triene-10,13-dicarboxylate 82



Aminomethyl polystyrene resin (80.0 mg, 0.745 mmolg^{-1}) was functionalised according to general procedure 1 then Fmoc deprotected and amino acid coupled with Fmoc-Lys(*endo*-BCN)-OH **75** according to general procedure 2. The resin (1 eq) was swollen in DCM for 5 min then washed with DCM (× 3). Diester dihydrotetrazine **45** (12.5 mg, 62.5 µmol, 1.05 eq) in DCM (0.595 mL) and DDQ (40.6 mg, 0.179 mmol, 3 eq) were added to the resin at rt. The resin was agitated for 4 h then filtered before washing with DMF (× 3), DCM (× 3), methanol (× 3) and diethyl ether (× 3). The linker was cleaved according to general procedure 3 to give crude product. The crude product was purified by flash column chromatography eluting with DCM up to DCM:methanol (49:1) to give *endo*-diester tricyclopyridazine derivatised Fmoc-Lys-NH_2 **82** as a colourless solid (37.0 mg, 87 %): **R$_f$** (DCM:MeOH, 19:1) 0.40; **mp** 178-179 °C; **IR** (neat, cm^{-1}) 3318 (N-H), 1682 (C=O), 1260 (C-O), 1156 (C-O); **^{1}H NMR δ** (500 MHz, $CDCl_3$) 7.74 (2H, d, *J* 7.5 Hz, Ar*H*), 7.57 (2H, m, Ar*H*), 7.38 (2H, t, *J* 7.4 Hz, Ar*H*), 7.29 (2H, t, *J* 7.5 Hz, Ar*H*), 6.26 (1H, br s, RCON*H*$_2$), 5.73-5.69 (2H, m, ROCON*H*R and RCON*H*$_2$), 4.86 (1H, br s, ROCON*H*R), 4.44-4.35 (2H, m,

RC*H*$_2$OCONHR), 4.20-4.17 (2H, m, R$_3$C*H* and ROCONHC*H*RCNH$_2$), 4.14-4.06 (2H, m, RC*H*$_2$OCONHR), 4.02 (6H, s, CO$_2$C*H*$_3$), 3.19-3.12 (2H, m, ROCONHC*H*$_2$R), 3.02-2.92 (4H, m, ArC*H*$_2$R), 2.34 (2H, br s, RC*H*$_2$CHR$_2$), 1.86 (1H, br s, RC*H*$_2$R), 1.67-1.51 (5H, m, RC*H*$_2$R), 1.39 (2H, m, RC*H*$_2$R), 1.10-1.07 (1H, m, R$_2$C*H*CH$_2$OCONHR), 0.84 (2H, br s, R$_3$C*H*); **^{13}C NMR δ** (125 MHz, CDCl$_3$) 174.3 (1C, RO*C*ONHR), 166.0 (2C, Ar*C*O$_2$R), 157.0 (1C, R*C*ONH$_2$), 156.5 (1C, RO*C*ONHR), 154.2 (2C, Ar), 143.8 (2C, Ar), 142.3 (2C, Ar), 141.4 (2C, Ar), 127.9 (2C, ArH), 127.2 (2C, ArH), 125.2 (1C, ArH), 125.1 (1C, ArH), 120.1 (2C, ArH), 67.1 (1C, R*C*H$_2$OCONHR), 62.2 (1C, R*C*H$_2$OCONHR), 54.4 (1C, ROCONH*C*HRCONH$_2$), 53.4 (2C, CO$_2$*C*H$_3$), 47.3 (1C, R$_3$*C*H), 40.3 (1C, ROCONH*C*H$_2$R), 31.8 (1C, R*C*H$_2$R), 29.5 (1C, R*C*H$_2$R), 27.5 (2C, Ar*C*H$_2$R), 23.3 (2C, R*C*H$_2$CHR$_2$), 22.4 (1C, R*C*H$_2$R), 18.8 (1C, R$_2$*C*HCH$_2$OCONHR), 18.5 (2C, R$_3$*C*H); ***m/z*** (ESI+, MeCN) 714.2 ([M+H]$^+$, 29 %), 736.2 ([M+Na]$^+$, 7 %), 317.3 (56), 102.3 (100); **HRMS** (ESI+, MeCN) [M+H]$^+$ found 714.3137 ($C_{38}H_{44}N_5O_9$ requires 714.3134); **HPLC** (column 3, method A, ELSD, MeCN) t_R 8.65 min.

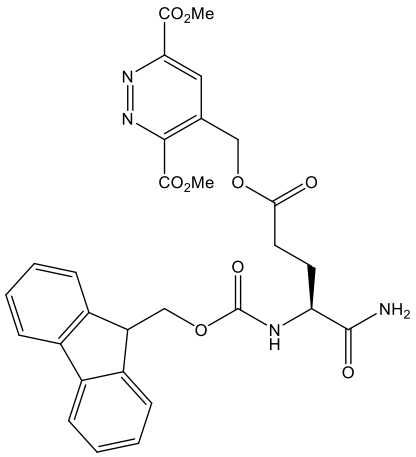
3,6-Dimethyl 4-({[(3*S*)-3-carbamoyl-3-{[(9*H*-fluoren-9-ylmethoxy)carbonyl]amino}propanoyl]oxy}methyl)pyridazine-3,6-dicarboxylate 84



Aminomethyl polystyrene resin (0.200 g, 0.745 mmolg^{-1}) was functionalised according to general procedure 1 then Fmoc deprotected and amino acid coupled with Fmoc-Asp(OAll)-OH according to general procedure 2. The resin (1 eq) was swollen

in DCM for 5 min then washed with DCM (× 3). Diester dihydrotetrazine **45** (31.2 mg, 0.156 mmol, 1.05 eq) in DCM (1.49 mL) and DDQ (0.101 g, 0.447 mmol, 3 eq) were added to the resin at rt. The resin was agitated for 4 h then filtered before washing with DMF (× 3), DCM (× 3), methanol (× 3) and diethyl ether (× 3). The linker was cleaved according to general procedure 3 to give crude product. The crude product was purified by flash column chromatography eluting with DCM up to DCM:methanol (49:1) to give diester pyridazine derivatised Fmoc-Asp-NH_2 **84** as a colourless solid (80.0 mg, 95 %): **R_f** (DCM:MeOH, 19:1) 0.32; **mp** 119-120 °C; **IR** (neat, cm^{-1}) 3336 (N-H), 1725 (C=O), 1685 (C=O), 1250 (C-O), 1138 (C-O); **^{1}H NMR δ** (500 MHz, $CDCl_3$) 8.35 (1H, Ar*H*), 7.73 (2H, d, *J* 7.6 Hz, Ar*H*), 7.56 (2H, d, *J* 7.4 Hz, Ar*H*), 7.40-7.36 (2H, m, Ar*H*), 7.31-7.27 (2H, m, Ar*H*), 6.35 (1H, br s, RCONH_2), 6.03 (1H, br s, RCONH_2), 5.70 (1H, br s, ROCON*H*R), 5.62-5.51 (2H, m, ArCH_2OCOR), 4.65 (1H, br s, ROCONHC*H*RCONH$_2$), 4.51-4.47 (2H, m, RCH_2OCONHR), 4.20-4.18 (1H, m, R_3C*H*), 4.07 (6H, s, CO_2C*H*$_3$), 3.16 (1H, d, *J* 15.7 Hz, RCH_2R), 2.87 (1H, dd, *J* 15.7 and 3.4 Hz, RCH_2R); **^{13}C NMR δ** (125 MHz, $CDCl_3$) 172.4 (1C, RO*C*ONHR), 171.3 (1C, RO*C*OR), 164.5 (1C, Ar*C*O_2R), 164.0 (1C, Ar*C*O_2R), 156.3 (1C, R*C*ONH_2), 152.5 (1C, Ar), 150.2 (1C, Ar), 143.7 (1C, Ar), 143.6 (1C, Ar), 141.5 (2C, Ar), 139.1 (1C, Ar), 128.0 (2C, ArH), 127.3 (1C, ArH), 127.2 (1C, ArH), 126.0 (1C, ArH), 125.1 (1C, ArH), 125.0 (1C, ArH), 120.2 (2C, ArH), 67.3 (1C, R*C*H_2OCONHR), 61.9 (1C, Ar*C*H_2OCOR), 53.8 (2C, CO_2*C*H_3), 50.9 (1C, ROCONH*C*HRCONH$_2$), 47.4 (1C, R_3*C*H), 35.7 (1C, R*C*H_2R); ***m/z*** (ESI+, MeCN) 563.0 ($[M+H]^+$, 23 %), 585.0 ($[M+Na]^+$, 34 %), 233.1 (28), 102.3 (100); **HRMS** (ESI+, MeCN) $[M+H]^+$ found 563.1777 ($C_{28}H_{27}N_4O_9$ requires 563.1778); **HPLC** (column 3, method A, ELSD, MeCN) t_R 7.43 min.

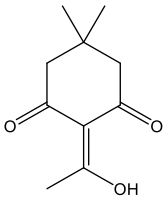
3,6-Dimethyl 4-({[(4*S*)-4-carbamoyl-4-{[(9*H*-fluoren-9-ylmethoxy)carbonyl]amino}utanoyl]oxy}methyl)pyridazine-3,6-dicarboxylate 85



Aminomethyl polystyrene resin (0.200 g, 0.745 mmolg^{-1}) was functionalised according to general procedure 1 then Fmoc deprotected and amino acid coupled with Fmoc-Glu(OAll)-OH according to general procedure 2. The resin (1 eq) was swollen in DCM for 5 min then washed with DCM (× 3). Diester dihydrotetrazine **45** (31.2 mg, 0.156 mmol, 1.05 eq) in DCM (1.49 mL) and DDQ (0.101 g, 0.447 mmol, 3 eq) were added to the resin at rt. The resin was agitated for 4 h then filtered before washing with DMF (× 3), DCM (× 3), methanol (× 3) and diethyl ether (× 3). The linker was cleaved according to general procedure 3 to give crude product. The crude product was purified by flash column chromatography eluting with DCM up to DCM:methanol (49:1) to give diester pyridazine derivatised Fmoc-Glu-NH_2 **85** as a colourless solid (74.0 mg, 86 %): **R_f** (DCM:MeOH, 19:1) 0.36; **mp** 124-125 °C; **IR** (neat, cm^{-1}) 1724 (C=O), 1692 (C=O), 1666 (C=O), 1257 (C-O), 1232 (C-O), 1137 (C-O); **^{1}H NMR δ** (500 MHz, $CDCl_3$) 8.29 (1H, Ar*H*), 7.74-7.72 (2H, m, Ar*H*), 7.56 (2H, d, *J* 7.3 Hz, Ar*H*), 7.39-7.35 (2H, m, Ar*H*), 7.31-7.27 (2H, m, Ar*H*), 6.31 (1H, br s, RCON*H*$_2$), 5.74 (1H, br s, RCON*H*$_2$), 5.65 (1H, br s, ROCON*H*R), 5.57-5.50 (2H, m, ArC*H*$_2$OCOR), 4.41 (2H, br d, *J* 5.9 Hz, RC*H*$_2$OCONHR), 4.32 (1H, m, ROCONHC*H*RCONH$_2$), 4.20-4.17 (1H, m, R$_3$C*H*), 4.07 (3H, s, CO_2CH_3), 4.03 (3H, s, CO_2CH_3), 2.74-2.53 (2H, m, RC*H*$_2$R), 2.30-1.92 (2H, m, RC*H*$_2$R); **^{13}C NMR δ** (125 MHz, $CDCl_3$) 173.3 (1C, RO*C*ONHR), 172.6 (1C, RO*C*OR), 164.5 (1C, Ar*C*O_2R), 164.1 (1C, Ar*C*O_2R), 156.5

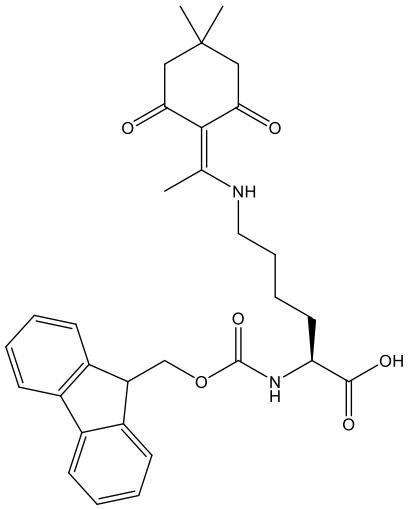
(1C, R*C*ONH$_2$), 152.4 (1C, Ar), 150.4 (1C, Ar), 143.8 (2C, Ar), 141.4 (2C, Ar), 139.4 (1C, Ar), 127.9 (2C, ArH), 127.2 (2C, ArH), 126.0 (1C, ArH), 125.2 (1C, ArH), 125.1 (1C, ArH), 120.2 (2C, ArH), 67.2 (1C, R*C*H$_2$OCONHR), 61.6 (1C, Ar*C*H$_2$OCOR), 53.8 (2C, CO$_2$*C*H$_3$), 53.5 (1C, ROCONH*C*HRCONH$_2$), 47.3 (1C, R$_3$*C*H), 30.1 (1C, R*C*H$_2$R), 28.2 (1C, R*C*H$_2$R); ***m/z*** (ESI+, MeCN) 577.2 ([M+H]$^+$, 32 %), 599.2 ([M+Na]$^+$, 52 %), 102.3 (100); **HRMS** (ESI+, MeCN) [M+H]$^+$ found 577.1915 (C$_{29}$H$_{29}$N$_4$O$_9$ requires 577.1935); **HPLC** (column 3, method A, ELSD, MeCN) t_R 7.46 min.

2-(1-Hydroxyethylidene)-5,5-dimethylcyclohexane-1,3-dione 88[129]



Acetic acid (8.59 mL, 0.150 mol, 1.05 eq) was added to a stirring solution of 5,5-dimethyl-1,3-cyclohexanedione (20.0 g, 0.143 mol, 1 eq), DMAP (18.3 g, 0.150 mol, 1.05 eq) and EDC (28.8 g, 0.150 mol, 1.05 eq) in DMF (300 mL) at rt. The reaction mixture was stirred for 1 d before the solvent was removed under reduced pressure. The residue was dissolved in ethyl acetate (200 mL) and washed with hydrochloric acid (3 × 200 mL, 1 M aq) and water (3 × 200 mL) then dried over anhydrous sodium sulfate before the solvent was removed under reduced pressure to give Dde-OH **88** as a yellow solid (20.9 g, 80 %): **R$_f$** (EtOAc:hexane, 1:9) 0.21; **mp** 34-36 °C; **IR** (neat, cm^{-1}) 1658 (C=O); **^{1}H NMR δ** (500 MHz, CDCl$_3$) 2.61 (3H, s, RCOHC*H*$_3$), 2.53 (2H, s, RC*H*$_2$COR), 2.36 (2H, s, RC*H*$_2$COR), 1.08 (6H, s, RC*H*$_3$); **^{13}C NMR δ** (125 MHz, CDCl$_3$) 202.6 (1C, R*C*OR), 198.1 (1C, R*C*OR), 195.4 (1C, R*C*OHCH$_3$), 112.5 (1C, RCO*C*RCOR), 52.6 (1C, R*C*H$_2$COR), 47.1 (1C, R*C*H$_2$COR), 30.8 (1C, R$_4$*C*), 28.7 (1C, RCOH*C*H$_3$), 28.3 (2C, R*C*H$_3$); ***m/z*** (ESI+, DCM) 183.1 ([M+H]$^+$, 17 %), 205.1 ([M+Na]$^+$, 17 %), 163.1 (96), 141.1 (35); **HPLC** (column 2, method A, ELSD, MeCN) t_R not observed. The spectroscopic data was in agreement with the literature.[129]

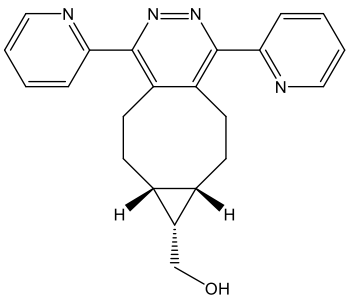
(2*S*)-6-{[1-(4,4-Dimethyl-2,6-dioxocyclohexylidene)ethyl]amino}-2-{[(9*H*-fluoren-9-ylmethoxy)carbonyl]amino}hexanoic acid 89



DIPEA (9.57 mL, 54.9 mmol, 1.5 eq) and Dde-OH **88** (10.0 g, 54.9 mmol, 1.5 eq) were added to a stirring solution of Fmoc-Lys-OH hydrochloride (14.8 g, 36.6 mmol, 1 eq) in methanol (350 mL) at rt. The reaction mixture was stirred overnight before the solvent was removed under reduced pressure. The residue was dissolved in ethyl acetate (250 mL) and washed with potassium bisulfate solution (250 mL, 1 M aq), hydrochloric acid (250 mL, 1 M aq) and water (250 mL) then dried over anhydrous sodium sulfate before the solvent was removed under reduced pressure to give crude product. The crude product was purified by flash column chromatography eluting with ethyl acetate:hexane (1:3) and ethyl acetate:methanol (9:1) to give impure product. The impure product was triturated in ethyl acetate:hexane (1:4) then dissolved in DCM:methanol (7:3) before the solvent was removed under reduced pressure to give Fmoc-Lys(Dde)-OH **89** as a colourless solid (14.4 g, 74 %): **R_f** (EtOAc:MeOH, 9:1) 0.65; **mp** 88-90 °C, literature 76-78 °C;[126] **IR** (neat, cm^{-1}) 3293 (N-H), 1714 (C=O), 1620 (C=C); **^{1}H NMR δ** (500 MHz, $CDCl_3$) 13.32 (1H, br s, RCN*H*RCH$_3$), 7.74 (2H, d, *J* 7.6 Hz, Ar*H*), 7.60-7.56 (2H, m, Ar*H*), 7.37 (2H, t, *J* 7.5 Hz, Ar*H*), 7.29-7.26 (2H, m, Ar*H*), 5.81 (1H, d, *J* 8.1 Hz, ROCON*H*R), 4.37-4.35 (1H, m, ROCONHC*H*RCO$_2$H), 4.34 (2H, d, *J* 7.2 Hz, RC*H*$_2$OCONHR), 4.19 (1H, t, *J* 7.2 Hz, R$_3$C*H*), 3.40-3.33 (2H, m, RNHC*H*$_2$R), 2.54 (3H, s, RCNHRC*H*$_3$), 2.35 (4H, s, RC*H*$_2$COR), 2.01-1.95 (1H, m, RC*H*$_2$R), 1.84-1.66 (3H, m, RC*H*$_2$R), 1.63-1.46 (2H,

m, RC*H*$_2$R), 1.00 (6H, s, RC*H*$_3$); **^{13}C NMR δ** (125 MHz, $CDCl_3$) 198.4 (2C, R*C*OR), 174.7 (1C, RO*C*ONHR), 174.2 (1C, R*C*NHRCH$_3$), 156.3 (1C, R*C*O$_2$H), 144.0 (1C, Ar), 143.9 (1C, Ar), 141.4 (2C, Ar), 127.8 (2C, ArH), 127.2 (2C, ArH), 125.3 (2C, ArH), 120.1 (2C, ArH), 108.0 (1C, RCO*C*RCOR), 67.2 (1C, R*C*H$_2$OCONHR), 53.6 (1C, ROCONH*C*HRCO$_2$H), 52.6 (2C, R*C*H$_2$COR), 47.3 (1C, R$_3$*C*H), 43.4 (1C, RNH*C*H$_2$R), 32.1 (1C, R*C*H$_2$R), 30.3 (1C, R$_4$*C*), 28.5 (1C, R*C*H$_2$R), 28.3 (2C, R*C*H$_3$), 22.6 (1C, R*C*H$_2$R), 18.3 (1C, RCNHR*C*H$_3$); ***m/z*** (ESI+, MeCN) 533.3 ([M+H]$^+$, 100 %), 555.3 ([M+Na]$^+$, 8 %); **HPLC** (column 2, method A, ELSD, MeCN) t_R 7.43 min. The spectroscopic data was in agreement with the literature.[140]

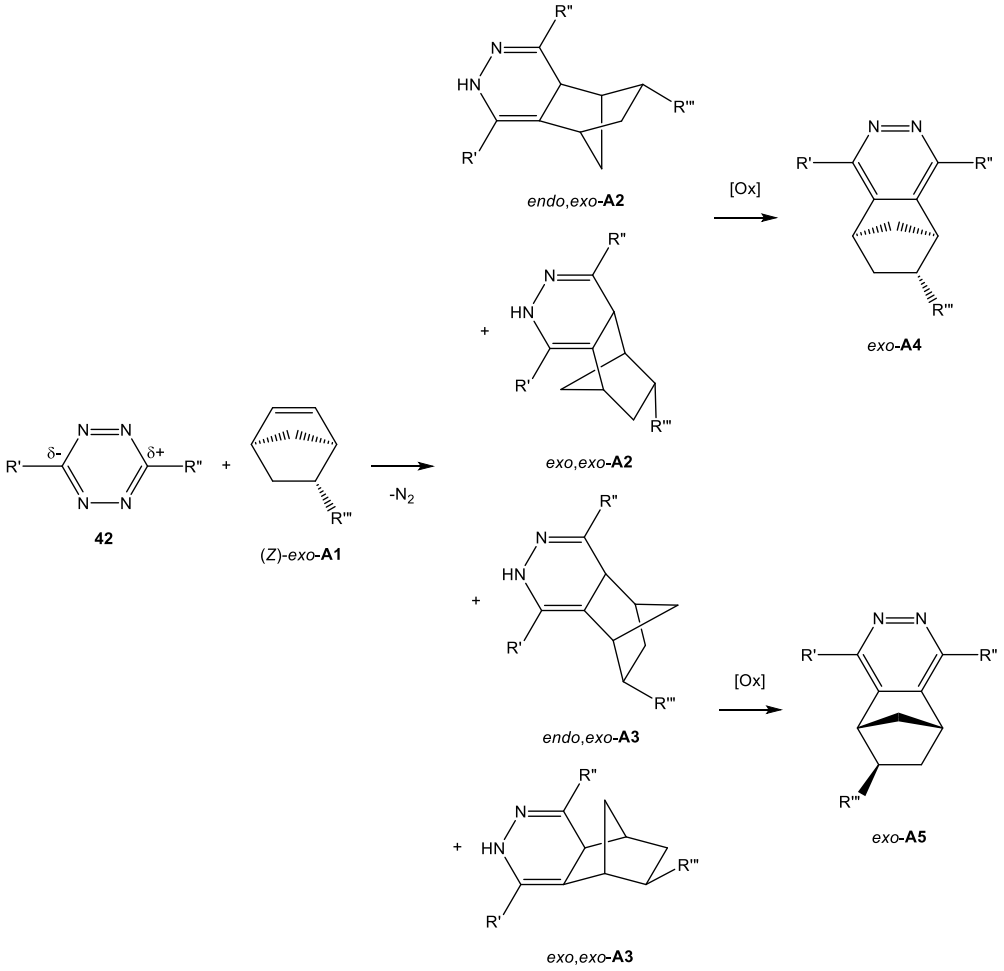
[(4*R*,5*S*,6*S*)-10,13-Bis(pyridin-2-yl)-11,12-diazatricyclo[7.4.0.0^{4,6}]trideca-1(13),9,11-trien-5-yl]methanol 93



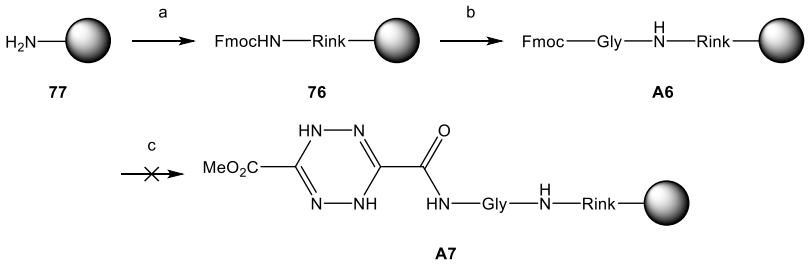
endo-BCN-OH **60** (44.0 mg, 0.293 mmol, 1.05 eq) was added slowly to a stirring solution of 3,6-di-2-pyridyl-1,2,4,5-tetrazine (65.9 mg, 0.279 mmol, 1 eq) in DCM (2.79 mL) at rt. The reaction mixture was stirred for 2 h before the solvent was removed under reduced pressure to give crude product. The crude product was purified by flash column chromatography eluting with DCM up to DCM:methanol (49:1) to give *endo*-dipyridyl tricyclopyridazine alcohol **93** as a colourless solid (94.0 mg, 94 %): **R$_f$** (DCM:MeOH, 19:1) 0.08; **mp** 223-224 °C; **IR** (neat, cm^{-1}) 3305 (O-H), 1034 (C-O); **^{1}H NMR δ** (500 MHz, $CDCl_3$) 8.72-8.71 (2H, m, Ar*H*), 7.95-7.94 (2H, m, Ar*H*), 7.87 (2H, td, *J* 7.7 and 1.8 Hz, Ar*H*), 7.37 (2H, ddd, *J* 7.5, 4.9 and 1.2 Hz, Ar*H*), 3.74 (2H, d, *J* 7.4 Hz, RC*H*$_2$OH), 3.17-2.90 (4H, m, ArC*H*$_2$R), 2.45-2.31 (2H, m, RC*H*$_2$CHR$_2$), 1.63 (2H, br s, RC*H*$_2$CHR$_2$), 1.48 (1H, br s, RO*H*), 1.18-0.99 (3H, m, R$_3$C*H* and R$_2$C*H*CH$_2$OH); **^{13}C NMR δ** (125 MHz, $CDCl_3$) 159.2 (2C, Ar), 157.2 (2C, Ar), 148.8 (2C, ArH), 142.3 (2C, Ar), 136.9 (2C, ArH), 125.1 (2C, ArH), 123.3 (2C,

ArH), 59.7 (1C, R*C*H$_2$OH), 27.9 (2C, Ar*C*H$_2$R), 24.4 (2C, R*C*H$_2$CHR$_2$), 22.8 (2C, R$_3$*C*H), 19.7 (1C, R$_2$*C*HCH$_2$OH); ***m/z*** (ESI+, MeCN) 359.2 ([M+H]$^+$, 74 %), 717.3 ([2M+H]$^+$, 5 %), 739.2 ([2M+Na]$^+$, 19 %), 317.2 (91), 102.2 (100); **HPLC** (column 2, method A, ELSD, MeCN) t_R 5.52 min. The spectroscopic data was in agreement with the literature.[123]

Appendix

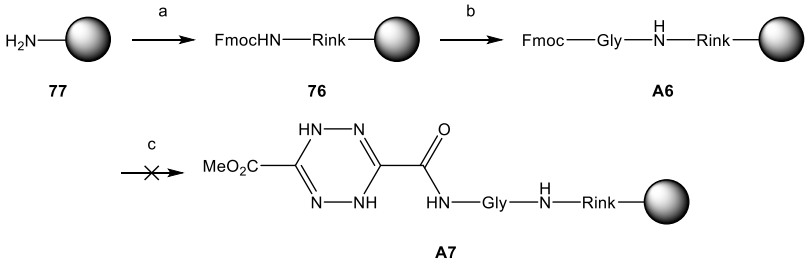


Appendix 1. iedDA reaction of electronically biased tetrazine **42** with electronically unbiased (*Z*)-*exo*-bicycloalkene **A1**.

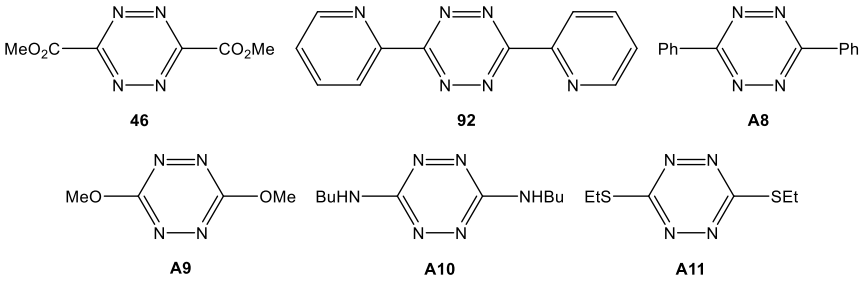


Appendix 2. Unsuccessful synthesis of resin-bound diester dihydrotetrazine derivatised H-Gly-OH **A7**. Reagents and reaction conditions a) Rink amide linker (3 eq), oxyma (3 eq), DIC (3 eq), DMF, rt,

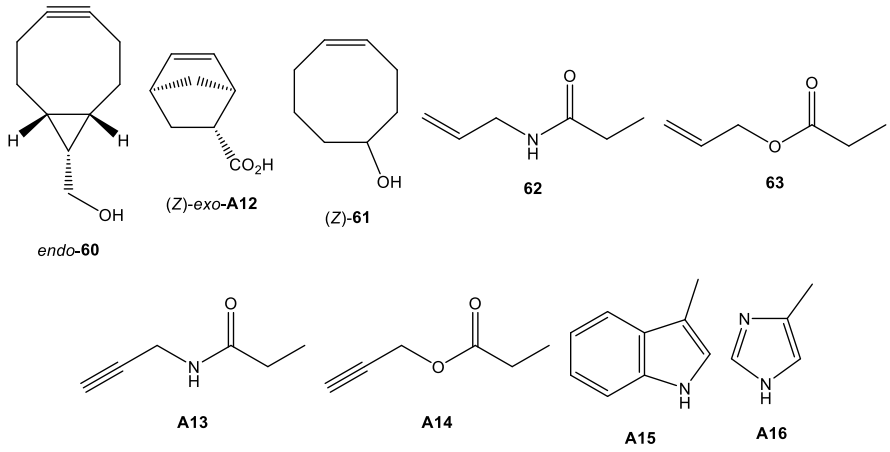
45 min; b) i) piperidine in DMF, rt, 5 min; ii) Fmoc-Gly-OH (3 eq), oxyma (3 eq), DIC (3 eq), DMF, rt, 45 min; c) i) piperidine in DMF, rt, 5 min; ii) diester dihydrotetrazine **45** (6 eq), DMF, reflux, 2 d.



Appendix 3. Unsuccessful synthesis of resin-bound diester dihydrotetrazine derivatised H-Gly-OH **A7**. Reagents and reaction conditions a) Rink amide linker (3 eq), oxyma (3 eq), DIC (3 eq), DMF, rt, 45 min; b) i) piperidine in DMF, rt, 5 min; ii) Fmoc-Gly-OH (3 eq), oxyma (3 eq), DIC (3 eq), DMF, rt, 45 min; c) i) piperidine in DMF, rt, 5 min; ii) dicarboxylic acid dihydrotetrazine **48** (3 eq), oxyma (6 eq), DIC (6 eq), DMF, rt, 45 min; iii) DCM:DIPEA:MeOH (80:5:15), rt, 45 min.



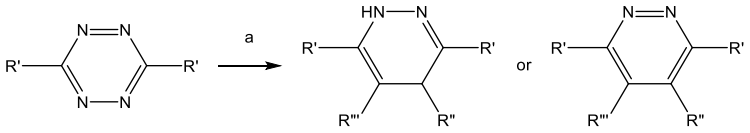
Appendix 4. 1,2,4,5-Tetrazine selection for the qualitative stability study of a library of 1,2,4,5-tetrazines and the qualitative screening studies of the iedDA reactions of a library of 1,2,4,5-tetrazines with a library of dienophiles.



Appendix 5. Dienophile selection for the qualitative screening studies of the iedDA reactions of a library of 1,2,4,5-tetrazines with a library of dienophiles.

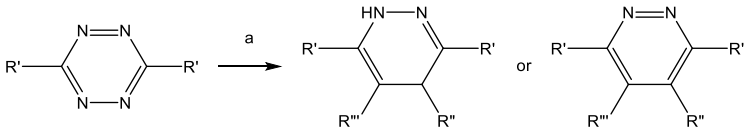
Entry	**1,2,4,5-Tetrazine**[a]	**Temperature**	
		rt	**60 °C**
I	**46**	< 30 min[b]	< 30 min[b]
II	**92**	> 2 d	> 2 d
III	**A8**	> 2 d	> 2 d
IV	**A9**	> 2 d	> 2 d
V	**A10**	> 2 d	> 2 d
VI	**A11**	> 2 d	> 2 d

Appendix 6. Qualitative stability study of a library of 1,2,4,5-tetrazines. [a] 0.1 M in DMF. [b] Complete decomposition of diester tetrazine **46** was indicated by a colour change from red to yellow.



Entry	Dienophile	1,2,4,5-Tetrazine[a,b]				
		92	**A8**	**A9**	**A10**	**A11**
I	*endo*-**60**	< 15 min	< 15 min	> 2 d	> 2 d	> 2 d
II	(*Z*)-*exo*-**A12**	< 30 min	< 1 d	> 2 d	> 2 d	> 2 d
III	(±)-(*Z*)-**61**	< 1 d	> 2 d	> 2 d	> 2 d	> 2 d
IV	**62**	> 2 d	> 2 d	> 2 d	> 2 d	> 2 d
V	**63**	> 2 d	> 2 d	> 2 d	> 2 d	> 2 d
VI	**A13**	> 2 d	> 2 d	> 2 d	> 2 d	> 2 d
VII	**A14**	> 2 d	> 2 d	> 2 d	> 2 d	> 2 d
VIII	**A15**	> 2 d	> 2 d	> 2 d	> 2 d	> 2 d
IX	**A16**	> 2 d	> 2 d	> 2 d	> 2 d	> 2 d

Appendix 7. Qualitative screening study of the iedDA reactions of a library of 1,2,4,5-tetrazines with a library of dienophiles at rt. Reagents and reaction conditions a) dienophile (1.05 eq), DMF, rt, 2 d. [a] 0.1 M. [b] Complete iedDA reaction of a 1,2,4,5-tetrazine with a dienophile was indicated by a colour change from purple to colourless.



Entry	Dienophile	1,2,4,5-Tetrazine[a]				
		92	**A8**	**A9**	**A10**	**A11**
I	*endo*-**60**	< 15 min	< 15 min	> 2 d	> 2 d	> 2 d
II	(*Z*)-*exo*-**A12**	< 30 min	< 4 h	> 2 d	> 2 d	> 2 d
III	(±)-(*Z*)-**61**	< 4 h	> 2 d	> 2 d	> 2 d	> 2 d
IV	**62**	< 8 h	> 2 d	> 2 d	> 2 d	> 2 d
V	**63**	< 1 d	> 2 d	> 2 d	> 2 d	> 2 d
VI	**A13**	> 2 d	> 2 d	> 2 d	> 2 d	> 2 d
VII	**A14**	> 2 d	> 2 d	> 2 d	> 2 d	> 2 d
VIII	**A15**	> 2 d	> 2 d	> 2 d	> 2 d	> 2 d
IX	**A16**	> 2 d	> 2 d	> 2 d	> 2 d	> 2 d

Appendix 8. Qualitative screening study of the iedDA reactions of a library of 1,2,4,5-tetrazines with a library of dienophiles at 60 °C. Reagents and reaction conditions a) dienophile (1.05 eq), DMF, 60 °C, 2 d. [a] 0.1 M. [b] Complete iedDA reaction of a 1,2,4,5-tetrazine with a dienophile was indicated by a colour change from purple to colourless.

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